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CONTENTS

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The Use of Labeled Amino Acids in the Study of the Protein Metabolism of Normal and Malignant Tissues: *A Review**

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From a biochemical point of view, cancer may be regarded as a metabolic disorder, characterized by a disturbance in the regulation of growth, with the production of autonomous cells. The statement is frequently made that there can be no single "cause" of cancer, but that it must be multiple, inasmuch as the number of known carcinogenic agents is so great. The question may be raised, however, whether the great variety of carcinogenic agents may not ultimately produce *the same metabolic effects* within the cell—by altering the biochemical machinery concerned in maintaining a balance in certain key synthetic and degradative mechanisms. From an operational standpoint, it may be useful to adopt this unitary point of view, which gives added incentive for an analysis of factors involved in the regulation of growth. Unfortunately, the problems of growth are almost infinitely complex, and if a common metabolic pattern characteristic of the neoplastic cell exists, it has not as yet been generally recognized.

The interrelations of protein, carbohydrate, lipid, and nucleic acid chemistry are being found to be increasingly numerous, and an alteration in the metabolism of one may likely produce an effect in all. Where, then, is the most promising point at which to begin a search for a metabolic distinction between normal and neoplastic tissue? In which parts of the biochemical field would a hypothetical metabolic change initiate the chain of events resulting in malignancy?

The scheme represented in Figure 1 expresses the idea that genes (considered here as either nuclear or cytoplasmic nucleic acid-containing materials concerned with hereditary transmission of biochemical characteristics) "direct" the synthesis of the apoenzyme or protein part of enzymes, which then becomes an active catalyst by combination with a coenzyme, in the presence of the proper ionic milieu. A large proportion of the enzymes within a cell of a higher animal is concerned with "metabolic housekeeping," but it is reasonable to suppose that certain enzymes are also concerned with the synthesis of genes. Many classes of cellular compounds have been shown to be synthesized, or their parts renewed, during the life span of a cell. One would wonder whether the chemical substances of which genes are composed also undergo replacement during the life of the resting cell. Direct evidence is lacking, but circumstantial evidence suggests (10, 22, 26) that desoxyribosenucleic acid may not undergo the constant renewal characteristic of many other cell constituents, but may only be formed during the time of cell mitosis. Such an arrangement would appear to provide for biochemical stability of the gene.

In the experiments of Bergstrand *et al.* (3), it was found that N^{15}-labeled glycine was built into the protein of the nuclei of resting rat liver as rapidly as it was into the protein of nuclei of regenerating rat liver. These data suggest that the protein part of the desoxyribosenucleo-protein (generally considered to make up a prominent part

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of the structure of the gene) behaves differently with respect to dynamic turnover from the desoxyribosenucleic acid portion. During mitosis the structure of the desoxyribosenucleic acids may conceivably be altered if a change has occurred in the enzyme proteins concerned with desoxyribosenucleic acid synthesis. *From this point of view, a carcinogenic agent may conceivably produce a hereditable change in the properties of a cell by acting on any one of a number of enzyme-catalyzed steps leading to construction of a gene.* The possibility of inducing a carcinogenic mutation would be enhanced, from this point of view, by increasing the mitotic rate of a tissue exposed to a carcinogenic chemical. This effect has, in fact, been reported (23, 34). That desoxyribosenucleic acid may have a mutagenic effect appears, of course, to be well shown by the experiments of Avery, McLeod, and McCarty (1). Evidence that a number of carcinogenic agents are also mutagenic has been forthcoming recently (18, 51, 52). A successful chemical carcinogen may then be a substance which has the two-pronged effect of stimulating cell division and of inducing chemical changes in the genes.

At our present primitive state of knowledge of the chemical factors involved in carcinogenesis, it may be premature, however, to consider a single avenue of biochemical study as being the most promising one. It may be more practical to take advantage of whatever new opportunities become available, in the hope that a definite clue turned up in any corner of the field may lead to a connection with the hereditary properties of the cell.

From clinical observations of the problem, one characteristic of malignant tissue appears to be its capacity to build new protein at a rate in excess of that of the normal tissue from which it originated. The search for an explanation of this accelerated synthetic rate may be considered a "second story" problem. The "first story" problem on which it rests is the development of an understanding of the mechanisms of protein synthesis itself. It is possibly too early to hope for an explanation of the increased rate of protein construction in the neoplasm, as long as the mechanism for the biological formation of a single new peptide bond is unknown. It may be possible, nevertheless, to minimize the time lag between the discovery of new knowledge in the field of protein metabolism and its application to the cancer problem.

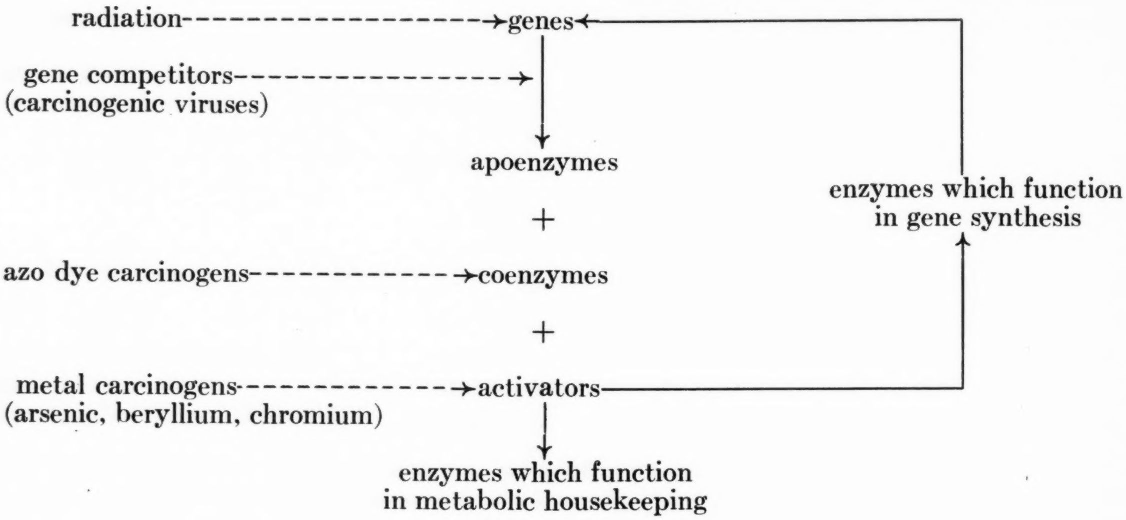

FIG. 1.—Possible sites of action of carcinogenic agents

THE FIRST STORY PROBLEM

Definition of terms.—Protein synthesis may be defined as a net increase in the mass of protein in a tissue, occurring during a particular period of time. If a labeled amino acid is injected into a growing animal, and if after a period of several days a large percentage of the labeled amino acid is found to be inextricably a part of the tissue protein of the animal, it may be assumed that this amino acid has taken part in the process of protein synthesis in the animal's tissue. It is quite possible, however, that the rate of uptake or turnover of the labeled amino acid in protein may even in this case exceed the rate of protein synthesis, as measured by the rate of increase in protein mass occurring during the experimental period.

The key problem still remains unsettled as to whether labeled amino acids become incorporated into protein structure solely by a process of "de novo" synthesis of the entire peptide chain, or whether the peptide chain can break at two peptide bond linkages and permit single amino acids

to be exchanged, with no net change in protein mass or structure occurring. *Upon the solution to this question rests the interpretation of all the results obtained with labeled amino acids in tissue slice and homogenate experiments and of a share of the results on whole animals.* In the absence of an answer to this question, the terms "incorporation" or "uptake" have been generally adopted as looser, more inclusive descriptions of the over-all process under study, encompassing the possibilities that the labeled amino acid may become part of a protein molecule in a biological system by (*a*) true "de novo" synthesis, (*b*) exchange, (*c*) reversal of proteolysis, or (*d*) combination in sulfhydryl or other unrecognized linkage different from the classical peptide bond. Vigorous efforts have been made, however (5, 36, 37, 57), to exclude the unpleasant possibility of a nonspecific adsorption of labeled amino acid onto protein as an explanation of the results. That protein synthesis to any biologically important extent may result from a reversal of proteolysis appears unlikely from an energetic standpoint (8, 19), but the possibility that incorporation of a labeled amino acid into protein to the extent of 0.1 per cent or less may occur by this mechanism has not been excluded.

One method of attack on the question of whether exchange occurs would be to add to a system such as a tissue homogenate several labeled amino acids and, then, to determine the specific activity of each in a pure, homogeneous protein isolated from the homogenate after the incorporation has taken place. If the rate of incorporation of each labeled amino acid into the protein were the same, the evidence would point toward synthesis of the entire peptide chain at one time. If the rates of incorporation were very disparate, however, it would suggest that a single amino acid might go into and out of peptide bonding in a protein independently of its neighbors. There are other possible attacks as well, such as partial hydrolysis of a pure labeled protein and determination of whether the specific activity of a single labeled amino acid is the same in various peptide fragments of the protein.

The only progress in this direction has come from the experiments of Simpson, Farber, and Tarver (48). These investigators found that ethionine not only inhibited the incorporation of S^{35}-labeled methionine into protein but also inhibited the incorporation of C^{14}-labeled glycine. It is difficult to explain this latter inhibition on an exchange basis. It is suggestive rather that the whole assembly-line of protein formation has been brought to a halt by the interference of ethionine with the incorporation of methionine into protein. It will be necessary, however, to determine whether ethionine interferes with incorporation of amino acids other than methionine and glycine before interpreting this result too broadly.

AMINO ACID METABOLISM OF NORMAL AND TUMOR TISSUE

The gateway to protein metabolism is the intracellular amino acid metabolism, and the door to the intracellular compartment is the cell membrane. It is therefore possible that regulation of the rate of protein synthesis may occur, in part, by way of the active transport mechanism of the cell membrane (15). Application of labeled amino acids to this problem has not yet been made to any great extent. Without the aid of isotopes, Roberts and Frankel (46) have, however, demonstrated that malignant tissues have a fairly characteristic free amino acid pattern, as determined by paper chromatography. In neoplastic tissues, the amino acids which are particularly abundant include glutamic and aspartic acids, which are also those that many tissues are capable of synthesizing, but which at least one malignant tissue can synthesize more rapidly than can its normal counterpart (20). It is conceivable that the concentration of certain key amino acids within the cell may be an important factor in limiting the rate of protein synthesis. These two amino acids are closely linked to carbohydrate metabolism, and their concentrations may reflect the concentrations of alpha-ketoglutaric and oxalacetic acids present at any time within the cell. When an abundant concentration of carbohydrate intermediates is available, formation of these amino acids may be favored. This way of thinking has the merit of suggesting that the rate of protein synthesis may be synchronized with an adequate rate of production of carbohydrate metabolites.

Van Pilsum and Berg (53) have recently shown that an excess of L-methionine, added to an otherwise balanced amino acid mixture, may retard the growth of rats. The thought thus emerges that an excess of a particular amino acid may serve as a built-in control on the rate of protein synthesis in the presence of an unlimited supply of amino acids. It is also reasonable to suppose that growth may be limited by the formation of inhibitors by the organism itself (45). Although it is difficult to adduce good evidence for the presence of such "internally produced inhibitors," this concept and its corollary, that a substrate for one enzyme may be a governor for another (17), provide springboards for experimentation in this direction.

Recent Studies on Protein Turnover in the Intact Animal and in the Perfused Organ

Among the interesting experiments in this category, only those of Bucher and Frantz (11)[1] and of Miller *et al.* (39) will be mentioned. The former investigators injected radioactive glycine into rats and found the half-life of the rat liver proteins, as measured by their concentration of radioactive glycine, to be 5.6 days. When another series of rats was given repeated injections of large amounts of nonradioactive glycine after injection of the radioactive glycine, the half-life of the liver proteins decreased to 2.4 days. This great reduction in half-life time did not occur when large amounts of alanine rather than glycine were used for "washing out." The implication of this experiment is that the turnover of protein in organs may be faster than experiments hitherto have led us to believe.

As a result of protein degradation, the labeled glycine may be liberated intracellularly, but under the usual experimental circumstances it would have a good opportunity of becoming rebuilt into new proteins. Where the pool of this particular amino acid is greatly augmented, however, there would be less chance of labeled glycine being caught up again in the synthetic process. Thus, it is worth considering half-life times, now generally available on proteins, as being maximal figures, which may be revised downward as more accurate methods of measuring the rate of the intracellular protein turnover become available. This point of view has been expressed previously by Rittenberg (44).

Miller and co-workers (39) perfused rat liver with C^{14}-labeled DL-lysine, and found it essential to have a balanced mixture of amino acids in the perfusion medium before appreciable incorporation of the lysine into protein took place. This is such a logical finding, and it fits so well with information gathered on the necessity of a complete amino acid mixture for protein construction in the intact animal (12), that it brings into relief the results of *in vitro* slice experiments. In the latter, incorporation of a labeled amino acid into slices of proteins takes place just as well if only one amino acid is added to the slice medium as it does if a synthetic mixture or an enzymatic hydrolyzate of protein is added.[2] In the latter case there may, however, be a sufficient intracellular concentration of amino acids already present to permit the incorporation to proceed. In the slice there apparently is sufficient proteolysis taking place to supply a well balanced amino acid mixture to the cell for synthetic purposes.

Incorporation of Labeled Amino Acids into *in vitro* Systems

Slices and isolated cells.—The advantage of the use of the tissue slice rather than the whole animal is in permitting more experimental variables to be kept under control. There is in this way a prospect of more rapid progress in uncovering factors which may influence the rate of incorporation and possibly, therefore, that of protein synthesis. In return for this advantage is imposed the risk that the protein metabolism of a surviving slice may provide a misleading picture of the pathways in operation in the intact animal.

There is general agreement (6) that both indispensable and dispensable amino acids are taken up *in vitro* by tissue proteins. In adult rat liver slices incubated in C^{14}-labeled alanine and S^{35}-labeled methionine, approximately 0.2–0.3 per cent of the alanine and methionine molecules present in the protein at the end of 4 hours' incubation were found to be labeled (37, 59). This would represent a rate of uptake of approximately 1.5 per cent per day for these amino acids. This rate is of the order of 5–10 per cent of that found in recent experiments on the rate of incorporation of alanine into whole rat liver (11). The slice experiments mentioned above were carried out under survival conditions, and it would be reasonable to expect that conditions for the incorporation process would be less favorable than those present in the whole animal.

In surviving slices, the incorporation rate has been found to vary with the type of tissue used and to be more rapid in slices from young than from old animals (24). Adult animal tissues which display the most rapid incorporation rates are intestinal mucosa (55) and bone marrow (6). It is well known that the cells of these tissues have a short life span and a high mitotic rate, implying the necessity for a high rate of protein synthesis if the tissue is to continue functioning. It has also been found (20) that the leukocytes present in human peripheral blood incorporate alanine and glycine into their proteins at a rate comparable to that found by Borsook for rabbit bone marrow. It is thus possible to study the rate of incorporation of amino acids into leukocyte protein under various experimental conditions. Evidence so far available indicates that little or no incorporation of labeled amino acids into non-nucleated or nonreticulated erythrocytes occurs. This would appear to imply that protein synthesis and turnover do not occur, or else they take place at a very slow

[1] N. L. R. Bucher and I. D. Frantz, Jr., unpublished data.

[2] P. C. Zamecnik and M. L. Stephenson, unpublished data.

rate in the adult red cell. In connection with this statement, it may be pointed out that although incorporation of labeled amino acids into protein may conceivably occur without "de novo" synthesis (i.e., by exchange), it is difficult to understand how protein synthesis can occur without the labeled amino acids becoming part of the newly formed protein.

In the slice work mentioned above, there was no evidence presented for net production of protein. A very pretty piece of work has recently been carried out, however, on chicken liver slices by Peters and Anfinsen (40). They demonstrated a net synthesis of a protein indistinguishable from chicken serum albumin, during a 4-hour incubation of the slices in a medium containing $C^{14}O_2$. The $C^{14}O_2$ was built into the carboxyl groups of glutamic and aspartic acids, and the latter were then incorporated into liver proteins. The liver slices secreted into the incubation medium a protein of higher specific activity (8–16 times as high as unfractionated liver proteins) than that of the liver slice protein as a whole. This protein was identical with chicken serum albumin by electrophoretic, ultracentrifugal, and immunological criteria. There was good evidence for a net synthesis of this albumin during the *in vitro* incubation of the slices. The increase in weight of the albumin in the system and the rate of incorporation of radioactivity into this albumin fraction also had parallel curves (41).

The homogenate.—One way of studying the relationship of cell architecture to metabolic processes is to smash up the tissue so that no intact cell membranes remain, with the result that particulate fragments lose their characteristic spatial relationship to each other. It would be anticipated that energy transport systems and synthetic assembly-lines would suffer and that catabolic processes would predominate. It is therefore of great interest that the uptake of labeled amino acids into the protein of essentially cell-free homogenates and fractions thereof has been reported (4, 5, 7, 24). The rate of incorporation of amino acids into homogenates has been found to be of the order of one-fifth to one-tenth of that found in tissue slices, over a 1–2-hour period, although the rates are more comparable over a 15-minute reaction period. The ability of the homogenate to incorporate glycine decays rapidly, while that of the slice remains relatively unimpaired over a period of hours. This lower total uptake in the homogenate brings results uncomfortably close to the level at which artifacts such as adsorption (36), co-precipitation (29), and combinations of amino acids in other than peptide linkage (54, 57) may be difficult to distinguish from bonding of amino acids in true peptide linkage in the protein.

The interpretation of what is going on in the homogenate incorporation studies is not as yet clear. When labeled lysine is incubated with a guinea pig liver homogenate, it becomes attached to protein whether oxygen is present or not (4, 5). When labeled glycine is used, the incorporation process is oxygen-dependent (24). A good share of the radioactivity which remains after washing-out of phosphatidyl serine can, however, be separated from the homogenate protein in this type of experiment, by suspension of the protein in thioglycolic acid.[3, 4] The possibility that glutathione or some other small peptide may contain the labeled glycine and be loosely attached to the larger protein moiety by sulfhydryl linkage is thus to be considered.

It may be fair to conclude that *the term "incorporation" covers more than one independent process by which amino acid and protein become closely associated in a chemical bonding.* The homogenate appears at present to be a biochemical bog in which much effort is being expended to reach firm ground.

Simpler systems.—In view of the difficulties involved in ascertaining whether a labeled amino acid has really become part of a protein molecule, it has appeared more profitable to certain investigators to set up as a more modest objective the study of factors involved in the biological synthesis of a single peptide bond.

Johnston and Bloch (27) have demonstrated that C^{14}-labeled glycine is incorporated into glutathione in acetone-dried powder made from pigeon liver. A dependence of this reaction on ATP as an energy source was originally shown. It has been found more recently, however (28), that this incorporation reaction may proceed without ATP, provided that glutamine, adenylic acid, and inorganic phosphate are present. Since an absolute increase in the amount of glutathione has not as yet been demonstrated unequivocally during the course of the experiments, it is possible that an exchange reaction may be taking place, with labeled glycine replacing inert glycine in glutathione, and little or no net glutathione synthesis taking place. It also brings to mind the coupled reaction sequences which Bergmann and Fruton (2) found to occur in peptides in the presence of catheptic enzymes, independently of an outside energy source. Thus, in a system simpler than that of whole protein, the problem again arises as to

[3] D. M. Greenberg, private communication.

[4] P. Siekevitz, unpublished data.

how to interpret the event recorded by the labeling process. It may be that the new ultraviolet absorption method of Racker (42) for measuring glutathione will facilitate determination of whether a net synthesis of glutathione occurs under these experimental conditions.

In the case of the formation of "peptidic" linkages (32), in which peptide-like bonds are formed in simple, two-component systems, the mechanism under scrutiny is more nearly understood. It has been shown that acetylation of sulfanilamide (33), the synthesis of glutamine from glutamic acid and ammonia (49), the condensation of *p*-aminobenzoic acid with glycine to form *p*-aminohippuric acid (16), Chantrenne's reaction involving an amino acylphosphate derivative (13), and similar systems (35) are all dependent on the energy-rich phosphate bond of ATP as an energy source. Here there is no question but that a net "de novo" peptidic bond synthesis has taken place, and the mechanism involved is for the first time clarified. The greatest penetration into the intricacies of energy donation and transfer in the formation of one of these peptidic linkages has been made by Lipmann, Novelli, and co-workers (14, 50), who have discovered the participation of a new coenzyme (A), and have dissected out several components of the system necessary for the acetylation of sulfanilamide. An interesting implication of these new findings is that the acetyl radical CH_3CO- may be transferred from combination in acetylphosphate to a temporary combination with coenzyme A, from which it is passed on to final combination with the amide group of sulfanilamide. The acetyl group-transferring function of the coenzyme introduces the concept that similar donor and acceptor mechanisms may operate in transferring reactive radicals in other synthetic processes. Thus, the mechanism of formation of a peptide-like bond is complex indeed, even when studied in a so-called simple model system. There is, furthermore, no assurance that the same energy transfer systems which operate in sulfanilamide acetylation are concerned in true peptide bond synthesis. It may be, however, that once initial peptidic bonds are formed by means of energy supplied from the outside, the so-called proteolytic enzymes may, by exchange reactions and coupled reaction sequences, then lengthen the peptide chain and impress on it the amino acid sequential characteristics of a particular protein species (21). The most important evidence pointing in this direction has come from the work of Brenner, Müller, and Pfister (9). These investigators report the synthesis of methionylmethionine and of methionylmethionylmethionine when methionine esters are incubated with chymotrypsin. The possibility is suggested that energy may be expended by way of phosphorylation to form amino acid esters, which can then be transformed into peptides by an exchange type of reaction. The latter is catalyzed by proteolytic enzymes without further introduction of outside energy. This explanation has the virtue of offering a mechanism for the introduction of energy into the formation of the peptide bond and of bringing into play the great specificity of the proteolytic enzymes as a means for arranging the sequence of amino acids in a peptide chain.

Protein Metabolism of Tumors

Outlining the problem clearly is of primary importance in undertaking a study of the protein metabolism of neoplastic tissue. The most succinct characterization of the nitrogen metabolism of malignant tissue has been given by Mider, Tesluk, and Morton (38), who conclude that *the tumor* (Walker carcinoma 256) *is a nitrogen trap*. This appears to be a fine point of embarkation for an investigation of the protein metabolism of malignant tissue. It implies that at least one tumor, as contrasted with normal tissues in the same animal, has a preferential mechanism either (*a*) for obtaining intracellularly the building blocks necessary for protein synthesis, (*b*) for getting along with simpler construction materials, (*c*) for keeping the protein assembly-line in action at a continuous rapid rate, or (*d*) for slowing down the proteolytic process. The questions to be pinned-to-the-wall are the following: are all these processes altered, or which step is changed; and is there any uniformity in the protein metabolism of neoplastic tissues as a class compared with non-neoplastic tissues?

Shemin and Rittenberg (47) have made a good beginning in this field. They fed N^{15}-labeled glycine *for 3 days* to rats bearing transplanted sarcoma R39. The rate of loss of labeled glycine from body tissues was then measured by sacrificing animals at intervals during the next 10 days. It was found that the tumor built labeled glycine into its proteins at a rate almost equal to that of liver but parted with it far less rapidly. The half-life of tumor protein was nearly twice as great (12 days) as that of liver (7 days). In the carcass (muscle and skin), however, the half-life of the protein was even greater than it was in the tumors. There was, therefore, a decreased rate of protein degradation in the tumor as compared with liver, but not as compared with muscle and skin. This type of study has been amplified, with employment of S^{35}-labeled methionine for greater sensitivity (30).

In such studies in the future, use of protein fractionation technics would provide greater information on differences in half-lives of particular classes of proteins in the normal and neoplastic tissues. It is possible that the structural proteins of the cell wall, or of the nucleus as contrasted with the cytoplasm, may give a more clear-cut half-life difference between tumor and normal tissue.

Griffin *et al.* (25) have made progress in this latter direction by studying the rate of incorporation of C^{14}-labeled glycine into rats bearing primary azo dye hepatomas. They found that the nonmalignant part of the livers built the labeled glycine into protein more rapidly than did the neoplastic part of the liver. The rate of loss of the radioactive label from the hepatoma protein was, however, slower than that from the nonmalignant hepatic tissue protein. The implication is that the half-life of the neoplastic protein is longer, or, expressed in another way, that the rate of protein degradation is decreased in the hepatoma as compared with nonmalignant liver tissue. In fractionation studies in which proteins of hepatic cell nuclei were isolated, this same finding was obtained—that a slower rate of uptake and of loss of radioactivity from the nuclei proteins and ribonucleoproteins of liver tumor cells occurred in comparison with noncancerous liver nuclei.

The problem of protein synthesis has also been investigated with whole animals and, in a few cases, with isolated tissues. Winnick, Friedberg, and Greenberg (56) injected C^{14}-labeled tyrosine into five rats. Two of these animals bore bilateral lymphosarcomas. Six hours after this single injection, one control rat and one tumor rat were killed. At 3 days another normal and tumor rat were killed. At 5 days the remaining normal rat was killed. The organs were worked up individually, and the rate of incorporation of C^{14} into the various organ proteins was then measured. Intestinal mucosa, kidney, and plasma exceeded the sarcoma in radioactivity contained per gram of protein, both at 6 hours and at 3 days. The radioactivity found in the proteins was accounted for almost entirely as tyrosine. The difficulties in obtaining sufficient C^{14}-labeled amino acid apparently restricted this early experiment to a very small number of animals.

A similar type of experiment was reported by Reid and Jones (43). Three mice bearing transplanted melanosarcoma S91 were given single injections of C^{14}-labeled tyrosine by tail vein. At 12, 24, and 72 hours a mouse was killed. The distribution of radioactivity among 23 separate tissues, as well as in urine and expired air, was studied. The tissues were dissected out, dried, and combusted, and the specific activity per milligram of dry tissue was recorded. Among the tissues with high specific activity were intestines, adrenal, thyroid, kidney, tumor, and plasma. Since protein was not separated from other constituents of the dried tissues, it is likely that labeled tyrosine was present in the various tissues in combination other than with protein. It is probable, however, that the incorporation rate of tyrosine into tissue proteins is very similar to that found for the dried tissue samples.

Le Page and Heidelberger (31) administered glycine-2-C^{14} by stomach tube to normal rats and to rats bearing transplanted Flexner-Jobling carcinomas. Animals were killed at 12, 24, and 48 hours. The specific activity of tumor protein was always higher than that of liver and thymus.

These studies described above testify to the fragmentary description now available of protein anabolism in neoplastic tissue.

Certain *in vitro* studies have also been carried out on the protein metabolism of tumors (58). In these experiments, slices from normal rat livers, nonmalignant portions of livers bearing azo dye-induced hepatomas, and from hepatomas were incubated in Warburg vessels for 3.5 hours in a medium containing either C^{14}-labeled alanine or glycine. The rate of incorporation of the labeled amino acids into the slice proteins was linear with time. Over a wide range of concentrations of added alanine, the rate of incorporation of labeled alanine into the hepatoma proteins exceeded that into control liver slice proteins (20). Since only 1 per cent or less of the alanine molecules present in the slice proteins was calculated to be newly formed during the time of the experiment, the authors inferred that a difference in the rate of protein degradation in the two types of slices could not explain the results. The conclusion was therefore reached that the hepatoma slice incorporated alanine and glycine more rapidly into protein than did either nonmalignant slices from the same livers or control slices.

The conclusions recently obtained by Griffin *et al.* (25) on whole animals bearing azo dye hepatomas do not appear to bear out these *in vitro* findings. It is possible that the slice experiments provide a test of the relative capacities of the neoplastic and normal hepatic tissues to incorporate the labeled amino acids into protein, under conditions in which an excess of the amino acid substrate is always available. This situation may not normally be present in the living animal.

SUMMARY

A reactivation of interest in studies on the mechanism of protein synthesis has come about as

a result of the availability of C^{14}-labeled amino acids. Although much information has been gathered, the crucial decisions as to whether phosphate bond energy is directly concerned in synthesis of the peptide chain, and whether the proteolytic enzymes participate, are still lacking. In application of the isotope technics to the cancer problem, no specific reaction has as yet been uncovered in which malignant tissue as a class differs qualitatively or quantitatively from all normal tissues. The dynamic aspects of the protein metabolism of tumors have thus far been the subject of only fragmentary studies. There appears to be a good opportunity for exploration with isotope technics of the property of tumors to continue to build new protein under dietary circumstances in which the remainder of the animal loses weight.

ADDENDUM

Since the above was submitted, two interesting articles have appeared, bearing on the new subject of transpeptidation.

Hanes, Hird, and Isherwood (Nature, **166**:288, 1950) present evidence that transpeptidation reactions involving glutathione can occur, catalyzed by crude enzyme preparations made from sheep kidney. They suggest that the stability of the γ-glutamyl linkage in glutathione against the action of peptidases may be of general importance in peptide metabolism within the cell. Johnston, Mycek, and Fruton (J. Biol. Chem., **185**:629, 1950) report that tissue cathepsins can catalyze transamidation reactions. They point out that a shift from pH 5, the optimum range for hydrolytic catalysis by these peptide-splitting enzymes, to pH 7.5 markedly increases the extent of transamidation and decreases the extent of hydrolysis. They suggest that at physiological pH values, transamidation (and presumably also transpeptidation) "may represent a major reaction in living cells; upon death of the cell, and the accompanying shift of pH to more acid values, the action of the intracellular proteolytic enzymes may be almost exclusively a hydrolytic one."

At the present moment it thus appears reasonable to consider protein construction as a two-step process. In the first step, phosphate bond energy is used for the formation of a few selected peptide bonds such as those in glutathione. Peptide chains may then conceivably be lengthened in the second step by means of transpeptidation reactions, which require enzymatic activation but little or no further expenditure of energy.

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A Transplantable Functional Ovarian Tumor Occurring Spontaneously in a Rat*

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Although the experimental induction of functional ovarian tumors in laboratory rodents, both by irradiation and by intrasplenic transplants, has been extensively studied, there are few records of spontaneous functional tumors in these animals. Spontaneous ovarian tumors of any variety are uncommon in rats. Bullock and co-workers (2, 4) found only six ovarian tumors in 14,038 female rats observed. We have been unable to find any report in the literature of a spontaneous functional ovarian tumor in the rat.

In the mouse, spontaneous ovarian tumors are also unusual. Slye and co-workers (8) observed only 44 ovarian tumors in 22,000 mice. Although Cloudman (3) referred to solid, spontaneous ovarian tumors, seen in the mice at Jackson Memorial Laboratory, as granulosa-cell tumors, Woolley, who is at the same laboratory, stated that none of these tumors showed evidence of function.[1] We have been able to find only two reports (Gardner *et al.* [6] and Strong *et al.* [9]) of spontaneous functional ovarian tumors occurring in mice.

It is the purpose of this paper to report a spontaneous functional ovarian tumor in the rat. This tumor displayed a high degree of endocrine activity and has grown readily on serial passage.

RESULTS

The tumor occurred in an adult female rat of the AXC line 9935. The animals of this line were originally obtained from Drs. Dunning and Curtis of the Detroit Cancer Institute and were maintained, in New Orleans, by line breeding. The animal had been set aside for timed pregnancy studies, and vaginal smears had been taken daily for 162 days. She had been caged with a male for the last 147 days of this period. The animal did not become pregnant, despite the occurrence of a vaginal plug. Cycles were never regular, diestrous smears predominating. During the last 43 days of observation, there was an unbroken sequence of diestrous vaginal smears. A mass was palpated in the flank, and the animal was killed with ether. The rat weighed 196 gm. at this time. A solid tumor measuring $2.6 \times 1.9 \times 1.9$ cm. (Fig. 1) had replaced the left ovary. There was congenital absence of the right horn of the uterus, a fairly common developmental anomaly in this line. The remaining horn was normally developed. The right ovary appeared atrophic.

The ovarian neoplasm was well encapsulated, and there was no evidence of local invasion or metastatic spread. It contained two cysts (3 and 5 mm. in diameter, respectively) which were filled with clear, yellow fluid. The tumor was composed of two types of tissue—one firm and brown, the other softer and almost white.

Whole mounts of the mammary glands were prepared. The development of the mammary glands was comparable to that seen on the twentieth day of pregnancy in this line (Figs. 2, 3). Grossly, the other organs appeared normal. The pituitary, which weighed 13.6 mg., was not enlarged.

Histologic observations.—The ovarian tumor was of the granulosa-cell type (Fig. 4), with an admixture of large lutein cells. Although granulosa cells were most conspicuous, theca cell areas could also be identified. Such histologic admixtures are also seen in induced neoplasms in rodents as well as in human ovarian tumors. The granulosa cells were arranged in varying histologic patterns: diffuse, trabecular, and papillary. Follicle-like structures, both large and small, were present. Some of the large follicle-like structures reproduced the normal architecture of the follicle to a remarkable degree, with a histologically distinct zone of theca cells

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[1] G. W. Woolley, personal communication.

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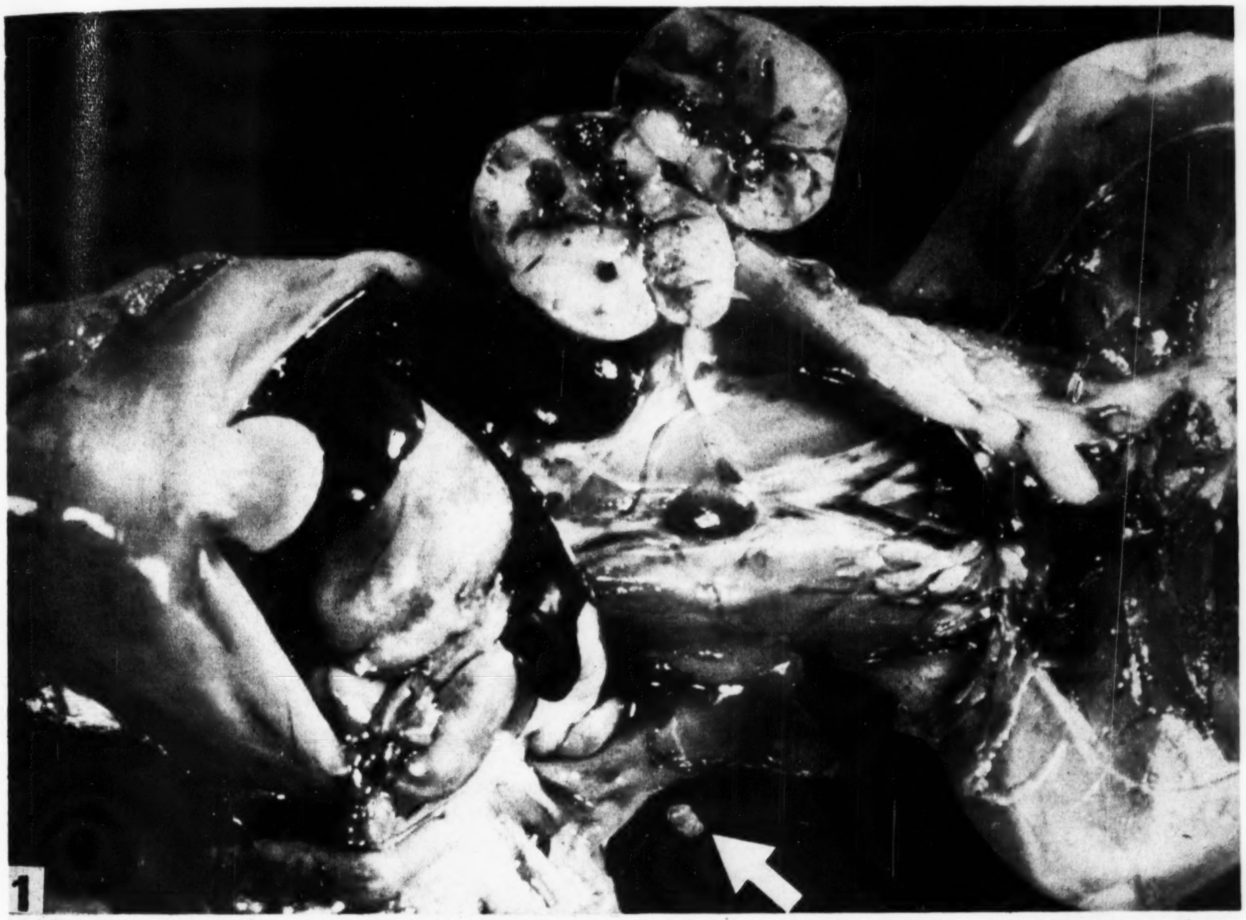
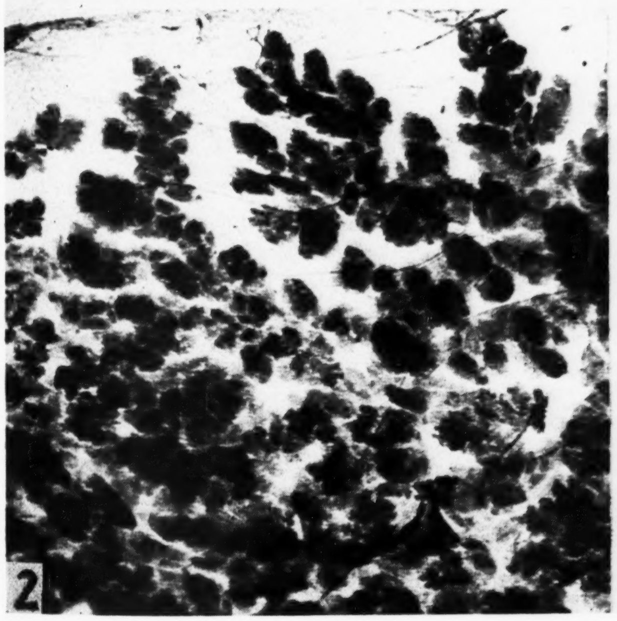




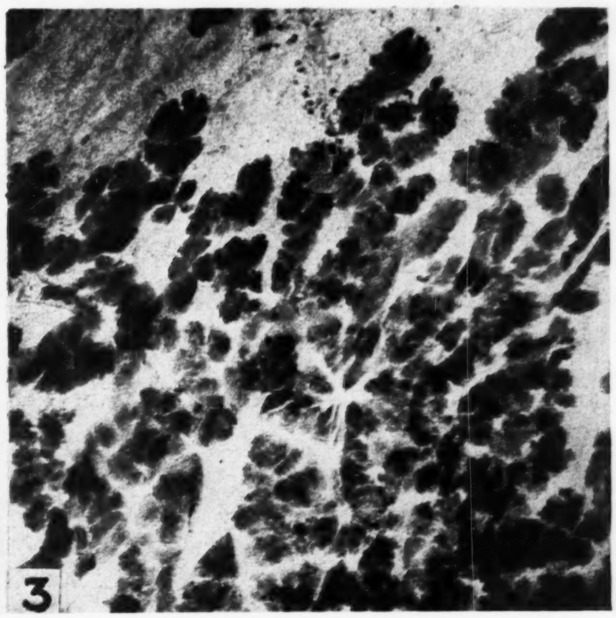
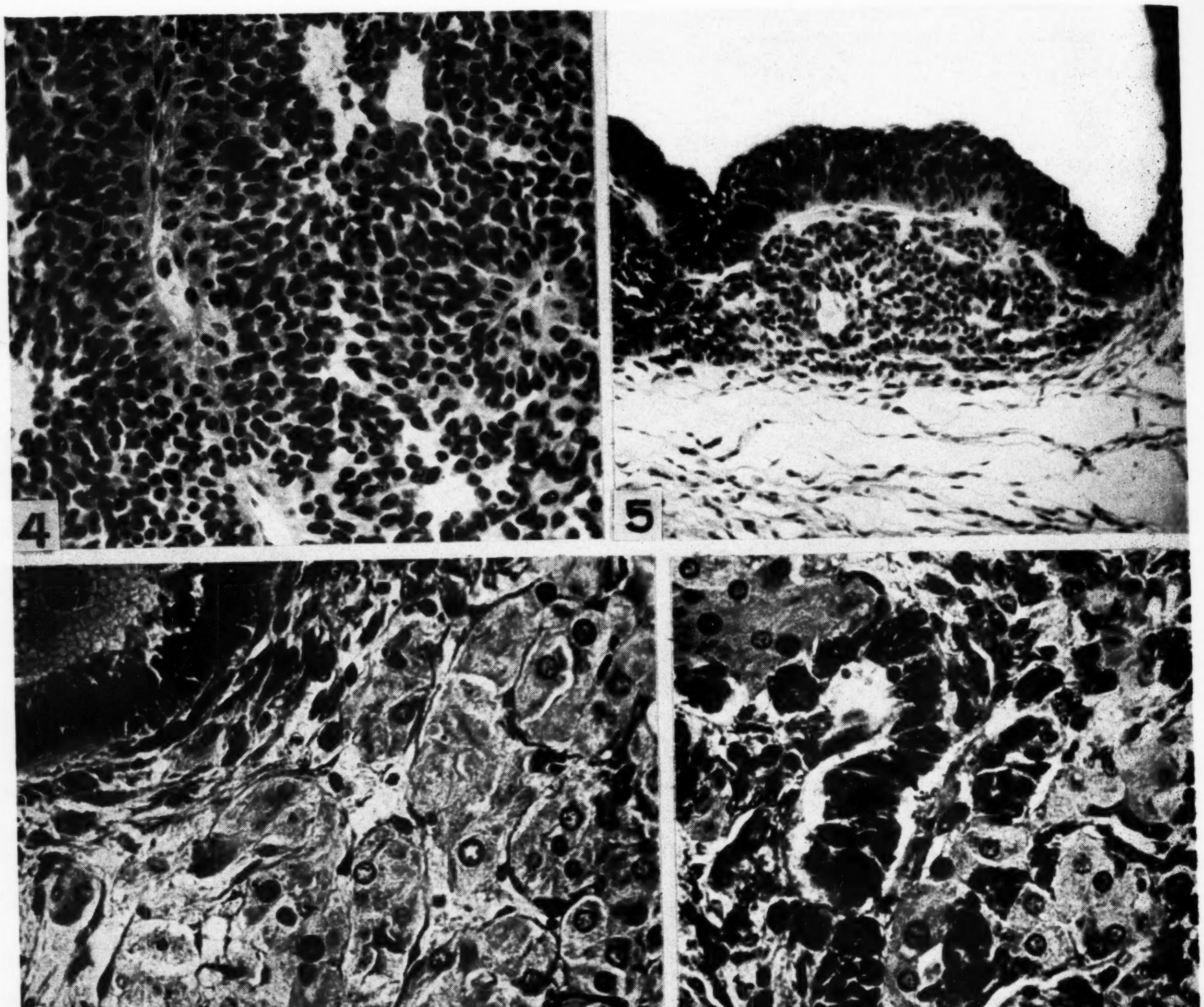


FIG. 1.—AXC rat, dissected at necropsy, showing spontaneous tumor of left ovary. The tumor (upper portion of photograph) has been sectioned and laid open. Arrow, lower, points to atrophic right ovary. There is congenital absence of right uterine horn.

FIG. 2.—Photograph of portion of cleared whole mount of mammary gland from AXC rat (above) bearing spontaneous ovarian tumor. The degree of mammary proliferation is equal to that of a term (20-day) pregnancy. ×12.

FIG. 3.—Photograph of portion of cleared whole mount of mammary gland from a control AXC rat at twentieth day of pregnancy. For comparison with Figure 2. ×12.



Figs. 4–9

surrounding the granulosa layer (Fig. 5). When stained for reticulum with silver, the theca cell layer showed a delicate reticulum about each cell, while the granulosa showed none—much as in the normal follicle.

The lutein cells were intimately intermingled with the granulosa cells but were strikingly different, histologically (Figs. 6, 7). The lutein cells had a diameter 4 or 5 times greater than that of the granulosa cells. The former had abundant, intensely acidophilic, granular cytoplasm, with a varying amount of vacuolization. They were arranged in interlacing cords within masses of granulosa cells; in some areas they were the predominant cell. The nuclei were more spherical, vesicular, and slightly larger than those of the granulosa cells, which were ovoid and hyperchromatic.

The granulosa cells had numerous mitoses, but none was noted in the lutein cells. It was not possible to ascertain whether the lutein cells were derived from granulosa or theca cells. Cells whose appearance suggested transitions to large lutein cells were seen among both granulosa and theca cells.

Cystic spaces containing blood or protein-rich fluid were abundant. Fibrosis, necrosis, granulation tissue, and foci of pigmented macrophages were conspicuous in and around the tumor.

Histologically, the uterus showed definite evidence of estrogen stimulation, although the response was probably not a purely estrogenic one. The vagina, showing extensive mucification of the epithelial cells, was similar to that seen in pregnancy. The mammary glands showed microscopic as well as gross evidence of proliferation, indistinguishable from that of late pregnancy. No significant microscopic changes were noted in the thyroid, adrenals, pituitary, skeletal muscle, spleen, kidney, liver, or thymus. Unfortunately, the right ovary was lost in the process of sectioning.

Transplants.—The original tumor was transplanted into six adult male and seven adult female rats of the same AXC strain. It was transplanted subcutaneously into two intact and two castrated male animals, and two other castrated males received intrasplenic grafts. Subcutaneous transplants were performed in three females. Four females, of which two were spayed, had intrasplenic transplants. In all six of the males and in three of the seven females the grafts grew progressively.

A total of 93 grafts (of which 62 were successful) were made over three transplant generations. The sites of transplantation, the number of successful transplants, and the gonadal status of the recipient animals are recorded in Tables 1 and 2. As indicated in Table 2, the number of successful transplants was greater in male recipients. A similar influence of sex has been observed in the transplant of induced granulosa-cell tumors in mice (1).

The histologic appearance of the tumor transplants in three serial passages did not differ significantly from the original tumor. In some the lutein cells were diminished in number, whereas occasional transplanted tumors showed a predominance of lutein cells. The granulosa cells reproduced the pattern of the initial tumor.

Only one metastasis was observed. This occurred in a castrated female rat in which the tumor had been implanted subcutaneously in the right axillary region. A bulky tumor grew at the site of transplantation, and a metastatic nodule in the cortex of the left kidney was observed at necropsy. On histologic examination, the metastatic nodule was composed of both granulosa and lutein elements.

Bone formation appeared in the stroma of several of the transplanted tumors. Such metaplastic bone has also been noted in transplants of experimentally induced granulosa-cell tumors (7). Histochemical studies of alkaline and acid phosphatase on several of the tumor transplants showed abundant alkaline phosphatase and little acid phosphatase in the granulosa cells of the tumor. It is likely that the production of bone in the transplants is related to the high alkaline phosphatase content of the tumor.

Evidence of hormonal production.—The functional character of the original tumor is indicated

FIG. 4.—Characteristic granulosa-cell pattern comprising bulk of spontaneous ovarian tumor pictured in Figure 1. Several mitoses are present. ×240.

FIG. 5.—Another section of the same ovarian tumor showing one of the larger follicle-like structures. The structure resembles the granulosa and theca cell layers of normal ovarian follicles. ×130.

FIG. 6.—Section of same tumor illustrating one of the luteomatous areas of the neoplasm. A follicle-like structure lined by granulosa cells is seen in the upper left. ×270.

FIG. 7.—Section of subcutaneous tumor growth following transplantation of fragment of original spontaneous ovarian tumor illustrated above into castrated adult female AXC rat. The transplanted ovarian tumor shows intermingling cords of granulosa and lutein-like cells. ×270.

FIG. 8.—Testis of adult AXC rat bearing subcutaneous transplant from the spontaneous ovarian tumor. There is profound atrophy due to estrogen production by the transplanted tumor with apparent inhibition of pituitary gonadotrophins. ×92.

FIG. 9.—For comparison with Figure 8. Testis of normal adult AXC control rat taken at same magnification. ×92.

by the mammary gland proliferation (equivalent to that of late pregnancy), comparable changes in the histology of the vagina, and irregular cycles as determined by vaginal smears. Diestrous smears were maintained for the 43 days before necropsy, and the animal failed to become pregnant.

There is little doubt that the tumor produces estrogens. Transplants of the tumor in spayed females resulted in pronounced proliferative changes in the mammary glands as well as (in some) estrous smears after a period of diestrus.

In several rats bearing transplants of this ovarian tumor for over 6 months, chromophobe adenomas of the pituitary developed. In two of these mammary adenocarcinoma also developed. Although spontaneous mammary carcinoma is rare in this strain, it is readily induced by estrogen (5). The occurrence of both pituitary chromophobe adenomas and mammary carcinomas is corroborative evidence of estrogen production by the tumor.

Subcutaneous tumor transplants in male rats produced pronounced atrophy of the testes (Fig. 8). The histologic appearance of the testis in these animals was comparable to that following hypophysectomy, presumably due to the inhibitory action of estrogen on the release of pituitary gonadotrophin. In addition, biologic assays for gonadotrophin were carried out on the pituitaries of three castrated rats bearing tumor transplants. Assays were accomplished by grafting such pituitaries into immature female rats. These preliminary experiments indicate that the presence of the transplanted tumor prevents the rise in pituitary gonadotrophin which ordinarily follows castration.

In males (intact and castrate) which received transplants of the tumor, the accessories showed a complex pattern which could not be interpreted as a pure estrogen effect. Seminal vesicles and coagulating glands resembled those of castrates to some extent, although the epithelium was much more abundant than that seen in the castrate state. Furthermore, there was none of the fibrous hyperplasia of the stroma that is seen with a purely estrogenic effect. The possibility remains that the tumor produces a hormone in addition to estrogen. However, a single attempt to demonstrate progesterone in a tumor-bearing animal, by the deciduoma test, yielded negative results.

TABLE 1

SUMMARY OF SERIAL PASSAGE TRANSPLANTS OF SPONTANEOUS FUNCTIONAL OVARIAN TUMOR IN 93 AXC RATS

	Female		Male		Total	
Serial passage	Rats implanted	Rats with tumor growth	Rats implanted	Rats with tumor growth	Rats implanted	Rats with tumor growth
1st	7	3	6	6	13	9
2d	33	21	34	21	67	42
3d	8	7	5	4	13	11
Total	48	31	45	31	93	62

TABLE 2

SUMMARY OF TUMOR TRANSPLANTS ACCORDING TO SEXUAL STATUS OF RECIPIENT ANIMALS AND SITE OF IMPLANTATION

	Subcutaneous transplants		Intrasplenic transplants		Intratesticular transplants		Totals	
Sexual status	Rats implanted	Rats with tumor growth	Rats implanted	Rats with tumor growth	Rats implanted	Rats with tumor growth	Rats implanted	Rats with tumor growth
Intact females	31	19	4	2			35	21
Castrate females	9	8	2	1			11	9
Intact males	22	20	3	2	7	1	32	23
Castrate males	6	5	9	4			15	9
Total	68	52	18	9	7	1	93	62

SUMMARY

A report of a spontaneous estrogen-producing ovarian tumor in an AXC rat is presented. The tumor was of the granulosa-cell type with an admixture of large lutein cells. It has been successfully transplanted over three transplant generations in male and female, intact and castrate rats. A single metastasis was observed, occurring in the kidney, and composed of both granulosa and lutein cells. There is abundant evidence for estrogen production by the tumor both in the original animal and in animals bearing transplanted tumors. Other hormonal production, although likely, has not been demonstrated conclusively. Pituitary adenomas were seen in rats bearing transplants of the tumor. In two of these animals mammary carcinoma also developed. Histochemical study indicated abundant alkaline phosphatase in the granulosa cells of the tumor. This may account for the oc-

currence of metaplastic bone in some of the tumor transplants.

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Heterologous Growth of Sarcoma 180 with Progression to Death of Hosts*

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Ehrlich was the first to demonstrate, by his famous "zig-zag" (i.e., mouse to rat to mouse, etc.) experiment, that the Ehrlich mouse sarcoma can grow in rats (4). Russell showed that a mouse tumor designated as "27" multiplied and survived in rats 9 days before regressing (12). Murphy demonstrated that mammalian tumors may be grown in the developing chick embryo (7). Murphy and Rous reported that rat tumor pulp injected through the uterine wall into the rat embryo, a few days prior to the end of pregnancy, became established but did not grow.[1] It is difficult to carry out this procedure without bringing gestation to an end (8). Bullock, using rats less than 24 hours old, succeeded in growing mouse Carcinoma 63 for 14 days and mouse Carcinoma 206 for 10 days before regression took place. Mouse Sarcoma 37 was maintained 49 days, with 5 passages, in baby rats (8). Gheorghiu, using rats not more than 3 hours old, was able to transplant a mouse carcinoma serially for 20 passages. The tumor regressed after growing 14 days in the first passage. Its longest period of growth occurred in the twentieth passage, where it was maintained for 28 days (5).

In all these experiments the tumors eventually regressed.

Murphy and Sturm (9) successfully transplanted mouse sarcoma into the brain of rats, guinea pigs, and pigeons and confirmed Shirai's finding (13) that the brain may be used for heterologous transplantation. Nagayo (10) transplanted sugar-induced spindle-cell mouse sarcoma serially in adult rats for ten generations. This procedure did not change the viability of the tumor cells, and there was no significant alteration of the tumor morphology. Putnoky (11) claimed to have grown the Ehrlich mouse sarcoma in lactic acid–treated adult rats for 26 passages. Greene (6), using the anterior chamber of the eye, has done extensive transplantation of heterologous tumors. Duran-Reynals (2, 3) has shown that a different disease may be produced when the Rous sarcoma virus was injected into either younger animals or animals of different species.

The establishment and growth of a tumor in foreign species is of interest not only in that it might be accomplished with human tumors but also in the study of tissue specificity. In the work to be presented, a study was made of the ability of the transplantable mouse Sarcoma 180 to grow in the rat, with special reference to the age of the rat and the route of inoculation.

MATERIALS AND METHODS

Cell suspensions were made by mincing a 7-day-old Crocker mouse Sarcoma 180 with a fine scissors and by adding equal parts of Locke-Ringer solution at pH 7.0. Various amounts of this cell suspension were inoculated into the rats (Table 1). Trocar pieces used in 1 part of the experiment were approximately 5 mg. each. In the serial passage (Table 2), 0.05 cc. of cell suspension was inoculated into all the animals. Rats of both sexes were used, which were litter mates, of the Sherman strain. The presence of metastatic lesions was verified by histological examination.[2] The viability of the tumor for the mouse was checked in the passage experiment by implanting portions of the rat passage tumor into Carworth or Rockland Farms white mice.

EXPERIMENTAL

Implantation of mouse Sarcoma 180 in rats of different ages.—Minced mouse Sarcoma 180 was implanted into rats of various ages by the intraperitoneal or subcutaneous route. The method used and the results obtained are summarized in Table 1.

It is apparent that minced mouse Sarcoma 180, injected intraperitoneally into rats 1–24 hours of age, proliferates readily and can cause the death of the animal within 7–13 days. At autopsy, large

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[1] Personal communication.

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[2] We wish to thank Dr. J. B. Thiersch of the Sloan-Kettering Institute for the microscopic examinations.

TABLE 1

SARCOMA 180 IN RATS OF VARIOUS AGES

AGE	NO. OF ANIMALS	METHOD OF INJECTION	DOSAGE	NO. OF TAKES	NO. OF REGRESSIONS	RESULTS Tumor	RESULTS No tumor
1–24 hrs.	3	I. P.	0.05 cc.	3		D7, 10, 10	0
1–24 hrs.	7	I. P.	0.1 cc.	7		D11, 11, 11, 13, 13, 13 K9	0
3 days	6	I. P.	0.05 cc.	6*	3	D13, 13, 17	K18, 18, 18
4 days	2	I. P.	0.1 cc.	2		K13, 13	0
12 days	6	I. P.	0.1 cc.	4†		K13, 13, 13, 13	K13, 13
21 days	7	I. P.	0.1 cc.	5‡		D15 K9, 9, 9, 9	K9, 17
51 days	9	I. P.	0.2 cc.	0		0	All K19
180 days	8	I. P.	0.2 cc.	0		0	All K20
1–24 hrs.	3	Subcu.	0.05 cc.	2	1	K7	K22, 22
1–24 hrs.	6	Subcu.	0.05 cc.	5	5	0	All K29
1–2 days	11	Subcu.	Trocar	8	8	0	All K14
7 days	7	Subcu.	0.1 cc.	1	1	0	All K20
180 days	9	Subcu.	0.2 cc.	0		0	All K20
180 days	10	Subcu.	0.2 cc.	0		0	All K15
180 days	5	Subcu.	0.2 cc.	0		0	All K10

D, day of death

K, day killed. Number following signifies the days after tumor inoculation that the animal was killed.

I. P., intraperitoneal inoculation.

* Tumor was palpable.

† Passage made to other 12-day-old rats. Killed 8 days later, no tumor seen.

‡ Passage made to 35-day-old rats. Killed 7–8 days later, no tumor seen.

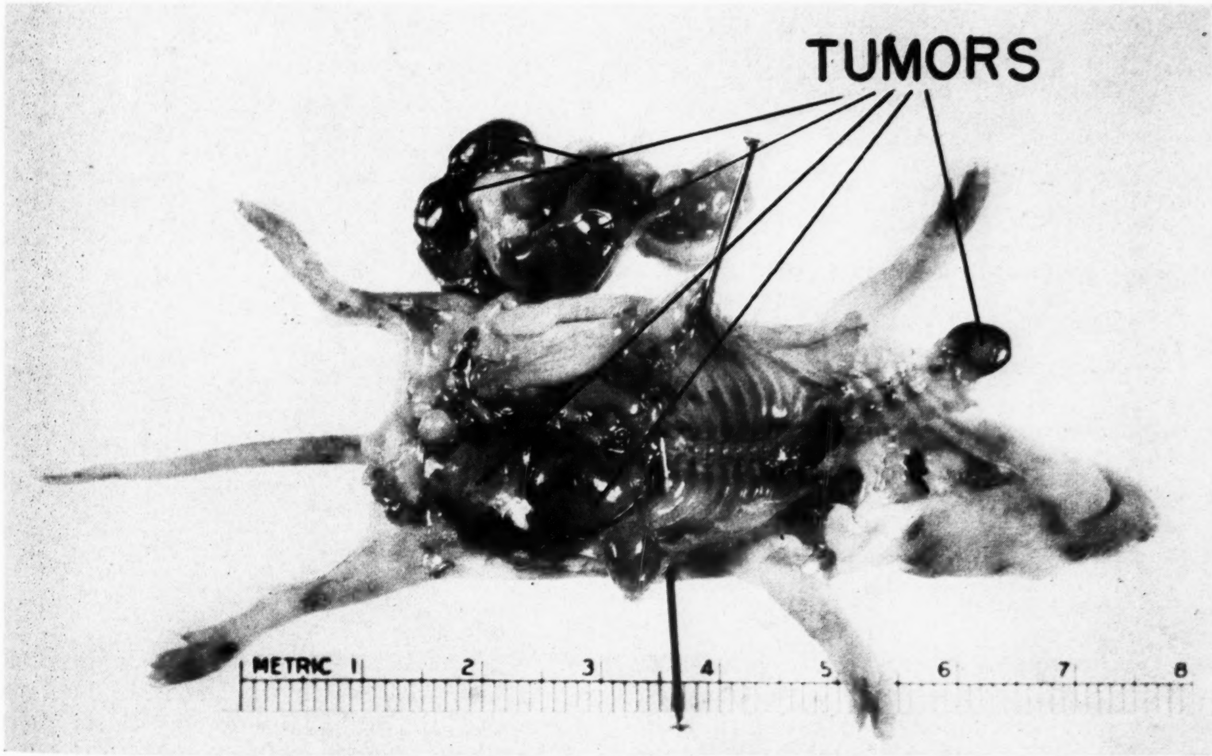


FIG. 1.—1–24-hour-old rat, dead 8 days after intraperitoneal injection of Sarcoma 180, showing tumor in abdominal cavity.

tumors were found in the peritoneal cavity and on the abdominal organs and on the xyphoid process (Fig. 1). Often the lungs were crowded with tumor nodules (Fig. 2). The invasiveness of the mouse tumor for the rat appeared to decrease progressively with the age of the rat, although successful implantations were possible up to 21 days of age. In younger rats (e.g., 3–4 days), widespread peritoneal implants occurred consistently if the animal died of the tumor, whereas those which survived showed regressing tumors when killed. Regressions were determined by palpating the abdomen at frequent intervals and noting the decrease in tumor size. The results were confirmed by autopsy and by finding the necrotic tumor.

When the Sarcoma 180 was injected subcutaneously into 1–2-day-old rats, the tumors grew for 7–10 days but then regressed. In older rats (180 days) no growth was noted.

Serial passage of the Sarcoma 180 in young rats.—Two attempts at serial passage in young rats were made. Minced tumor suspension was inoculated intraperitoneally. When the tumors were palpable, the animal was killed, the tumor removed and minced, and inoculated into other rats. Rats of various ages received both intraperitoneal and subcutaneous inoculations in an attempt to discover if the Sarcoma 180 could be adapted to an older host. In addition, mice were injected subcutaneously with the minced tumor, to determine if growth in the rat had interfered with the ability of the tumor to grow in its natural host. Microscopic examination was made of all tumor tissue and of tissue showing tumor nodules. Since the results of both experiments were identical, the details of only one of them are presented in Table 2.

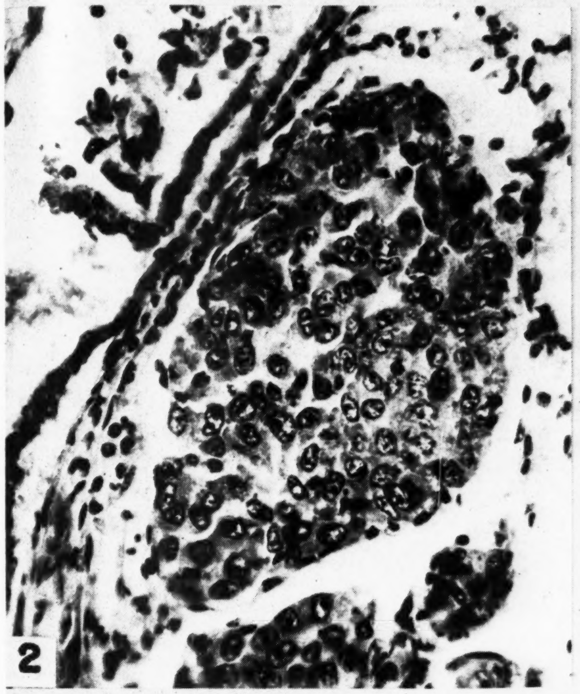
Continuous passage was possible only when rats of 1–2 days of age were used. In the first ex-

TABLE 2

SERIAL PASSAGE OF SARCOMA 180 IN RATS

Passage	No. of rats	Age	Method of injection	No. of takes	No. of regressions	Results: Tumor	Results: No tumor
1st	2	4 days	I. P.	2		K9, 9	
2d	8	2 days	I. P.	5		K8, 8, 8, 8, 8	K8, 8, 8
3d	8	1–24 hrs.	I. P.	6		D13, 14, 17 K16, 21, 21	K28, 28
4th	7	1–24 hrs.	I. P.	7		D11,12, 12, 14 K8, 8, 15	
5th	3	1–24 hrs.	I. P.	3		D10, 11 K7	
	4	2 days	I. P.	2		K14, 14	K52, 52
6th	10	1–24 hrs.	I. P.	10	2	D10, 10, 10, 12, 17 K10, 10, 10	K29, 29
7th	6	1–24 hrs.	I. P.	6		D11, 12, 14, 14, 14 K14	
8th	4	2 days	I. P.	1		K16	K22, 22, 22
	4	2 days	I. P.	3		D8, 10, K22	K22
	4	2 days	Subcu.	4	2	D15, K5	K22, 22
9th	6	1–24 hrs.	I. P.	5		K7, 7, 12, 12, 12	K12
	8	1 day	I. P.	4		K7, 7, 7, 7	K7, 7, 7, 12
	10	4 days	I. P.	8		K10, 10, 10, 10, 11, 11, 11, 11	K11, 11
	6	3 days	Subcu.	6	6		All K10
10th	8	2 days	I. P.	1		K8	K8, 8, 8, 8, 8, 8, 8
	3	1 day	I. P.	2		K8, 8	K8
	5	1 day	I. P.	1	1		All K8
11th	10	1–24 hrs.	I. P.	2		K13, 13	K13, 13, 13, 13, 13, 13, 13, 13
12th	8	2 days	I. P.	2		K7, 7	K24, 24, 24, 24, 24, 24

D, died of tumor.
K, killed. Number following signifies the days after tumor inoculation that the animal was killed.
I. P., intraperitoneal inoculation.



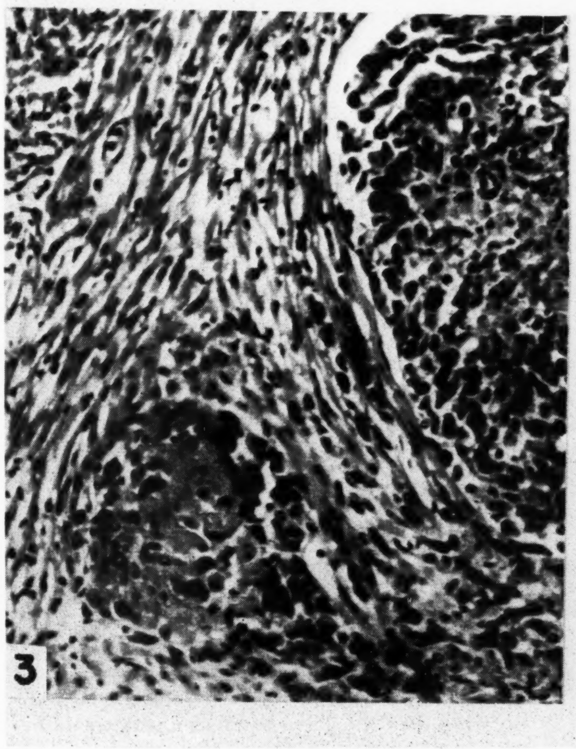
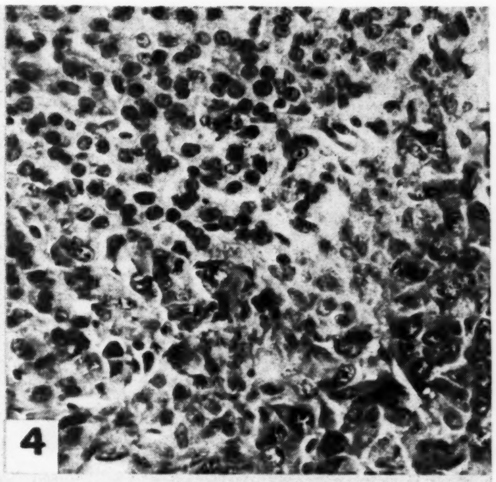
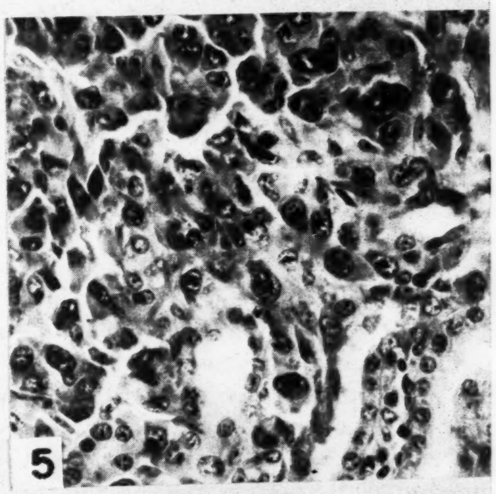


FIG. 2.—Metastatic nodule of Sarcoma 180 in lung of rat.
FIG. 3.—Metastatic nodules of Sarcoma 180 in heart of rat.





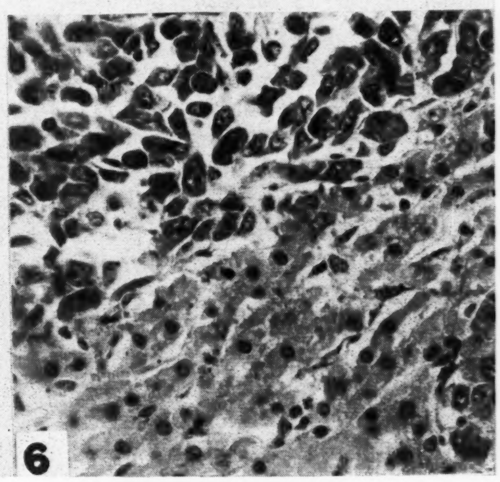


FIG. 4.—Invasion of rat adrenal by Sarcoma 180.
FIG. 5.—Invasion of rat kidney by Sarcoma 180.
FIG. 6.—Invasion of rat liver by Sarcoma 180.

periment, seven continuous passages were made before the experiment was abandoned because of the lack of rats of the proper age. In the second experiment, twelve successful passages were made. There was some evidence that the invasiveness of the Sarcoma 180 for the young rat had decreased somewhat, as measured by the amount of tumor growth and by the incidence of lung metastases. The tumor appeared to maintain its vigor for the foreign species for at least seven passages. After that period, there appeared to be a smaller number of successful takes and fewer distant metastases. However, if the passages had been made at shorter intervals, it might have been possible to continue the transfers indefinitely.

Microscopic examination of the tumors showed them to be typical Sarcoma 180, and there was no evidence that these tumors had changed histologically in the rats. An unexpected finding was the ability of the tumor to metastasize to the lung. This was found in many of the rats of the first six passages. In addition, there were often tumor nodules in the mediastinum, and on one occasion the heart muscle contained tumor (Fig. 3). The peritoneum was frequently filled with tumor masses with implantation and invasions of the liver, pancreas, kidney and adrenal (Figs. 4–6). Implantation of tumor on the xyphoid process was also noted in many instances.

There was no evidence that the Sarcoma 180 had lost its capacity to grow in the mouse, even after twelve passages in the rats. Histologically, it appeared unchanged.

DISCUSSION

It has been demonstrated that it is possible to grow a transplantable mouse tumor, the Sarcoma 180, in a foreign host—the rat—provided that the host is very young and that the route of inoculation is intraperitoneal. Under these conditions, serial passages can be made. It is probable that the peritoneal cavity of the newborn behaves in a similar manner to tissue culture or to the chorio-allantois of the chick, where heterologous transplants are successful. It appears that the factors responsible for the host specificity of tumors have not had a chance to develop in the very young animal. That such is the case is indicated by the almost uniform failure of the Sarcoma 180 to grow in older rats.

The fact that the rat passage tumor did not change histologically and that it retained its ability to grow absolutely normally in the mouse leads one to believe that the factors responsible for the failure of growth are more intimately connected with the heterologous host than with any inherent change in the tumor.

The vigor of the tumor growth in the foreign species was well demonstrated by the extensiveness of the implantation in various organs and the presence of distant metastases.

SUMMARY

It has been possible to make serial passages of the mouse tumor Sarcoma 180 in the newborn rat by utilizing the intraperitoneal route of inoculation. Subcutaneous inoculations of newborn rats and a transplant of the same tumor in older animals generally failed to grow or regressed after a short period. When inoculated into 1–2-day-old rats, the Sarcoma 180 generally brought about the death of the animal in a week, often with widespread metastases.

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Estrone Conversion Capacity of Blood of Postmenopausal Women with Carcinoma of the Breast*

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During the investigation of the fate of estrone in blood and perfused organs, it was found that estrone was converted to a biologically inactive substance (1), which could no longer be assayed as a 17-ketosteroid (2). Utilization of this latter fact in a study of the conditions under which this conversion could occur provided evidence supporting the hypothesis that an enzyme catalyzing the reaction is present in blood (2).

A number of blood samples from human female donors were found inactive in inducing the conversion. In each instance the donor had passed the menopause. Several blood samples from postmenopausal patients with carcinoma of the breast were found capable of inducing the conversion. The study to be reported was undertaken to determine grossly the extent of the divergence between normal postmenopausal women and those with carcinoma of the breast.

METHODS

Two normal young women were used as the control sources of blood. Checks on the procedure, with their blood, were requisite, because it was found that the adequacy of the White's solution as a diluent was subject to considerable variation even when freshly made. The variation was usually traced to presumed degradation of the vitamins in the White's solution. Thus, if a series of tests on specimens was run in which no estrone loss occurred, those tests were repeated using the blood from the control women at the same time to test the efficacy of the White's solution.

It was found that with these precautions the technic described below provided a sufficient degree of consistency for the study. Because of the laborious procedure, an all-or-none comparative assay technic, rather than one based on unit determination, was employed. The method in its final form was as follows:

1. Exactly 5 cc. of venous blood was diluted with 2 cc. of 0.07 M KCN solution. The resulting 7 cc. of 0.01 M KCN laked and diluted blood was thoroughly shaken while clotting proceeded. For the clinical work, the 2 cc. of 0.07 M KCN was prepared in rubber-capped vials. The blood was injected through the cap into the cyanide solution. This treatment of the blood effectively prevented growth of microörganisms, and on storage in the icebox the sample retained activity for 4-week periods.

2. One-, 0.5-, or 0.2-cc. samples of the diluted blood were added to 50 cc. of White's solution containing KCN at 0.01 M concentration (see below reasons for choice of these blood concentrations).

3. The mixture of blood and White's solution was placed in a water bath at 37.5° C. for $\frac{1}{2}$ hour. Four milligrams of estrone, dissolved in 13 cc. of saline solution (2), was then added to the reaction mixture. At precisely $\frac{1}{2}$ hour after the addition of the estrone, 50 cc. of chloroform was added to the vessel, and the flask was shaken violently for at least 30 minutes.

4. Ten cc. of the clear chloroform solution obtained after centrifuging the resulting emulsion was drawn off and evaporated to dryness. To the residue was added 2 cc. of ethyl alcohol. When the residue was completely dissolved, 50 cc. of 1 N NaOH was added. The resulting solution (which occasionally showed turbidity) was kept at room temperature for 5 minutes before being placed in an ice bath and made acid to phenolpthalein by the addition of CO_2.

5. After acidification the solution was extracted 4 times with $\frac{1}{4}$ volume of ether. The ether was washed with H_2O to remove traces of bicarbonate and was then evaporated to a water- and ether-free residue. The residue was treated as described earlier, and the phenolic ketonic portion obtained after the Girard separation was assayed polarographically (2).

Assay criteria.—Five control runs of the experiment by the technic described above gave re-

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covery values of 3.75 mg. of estrone when 4 mg. had been added to 50 cc. of White's solution as described. Because the White's solution did not contain the amount of protein which the blood added to the reaction mixture, and also to allow for the random errors inherent in routine assay procedures, a loss of 0.5 mg. out of the 4 was considered as possibly due to chance. Thus, recovery values of 3.4 mg. or less were considered as significant and indicative of the presence of estronase.

TABLE 1

ESTRONASE IN BLOOD FROM WOMEN WITH BREAST CANCER COMPARED WITH THAT FROM NORMAL WOMEN

Blood donor	No. of donors	Mean age (standard deviation and range)	No. showing "estronase" activity	No. showing no measurable "estronase" activity
Normal	19	54 ± 8.7	3	16
Breast cancer	14	66 ± 8.7	11	3

The volumes of 1.0, 0.5, and 0.2 cc. of diluted blood, as mentioned above, were employed, because most normal postmenopausal blood specimens contained insufficient enzyme to show an effect at a higher dilution than 0.5 cc. in 50 cc. of White's solution. Some were inactive at much higher blood concentrations. The dilutions employed as described seemed, however, to be critical for most specimens. Many specimens showed activity at 1 cc. per 50 cc., few at 0.5 cc. per 50 cc., and only one at 0.2 cc. per 50 cc.

RESULTS

The data for the comparison between normal postmenopausal women and those with carcinoma of the breast are presented in Table 1. The patients are compared at the 0.5-cc. dilution level. In the table are presented the number of patients in each group, the average age of each group, the age range, and its standard deviation. The results of the assays are presented as to both the number of patients in each group showing activity and those showing no activity.

From the results it is apparent that the postmenopausal women with carcinoma of the breast are very liable to retain in their blood a capacity to convert estrone which normally should disappear at the menopause. The relationship between this finding and the now well recognized therapeutic value of estrogens in inhibiting the growth of this particular malignancy should prove of interest.

SUMMARY

A semi-quantitative test of the estronase concentration in human blood has been described. The test was employed in a comparison of the titer of normal postmenopausal blood and that of postmenopausal women with carcinoma of the breast. The data show that the postmenopausal women with carcinoma of the breast can be expected to have a significantly higher blood titer of the enzyme than do normal postmenopausal women.

ACKNOWLEDGMENTS

Samples of postmenopausal blood were obtained from the Endocrine Clinic of the Beth Israel Hospital in Boston, through the courtesy of Dr. S. Gargill. The breast cancer blood samples were made available through the kindness of Dr. I. T. Nathanson of the Huntington Memorial Division of the Massachusetts General Hospital, Boston, and Dr. D. Hight of the Memorial Hospital, Worcester.

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The Polysaccharide Content of Serum Fractions in Carcinoma, Arthritis, and Infections*

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A rise of the polysaccharide associated with serum proteins has been noted in malignancy and in several other conditions (7). Seibert, Pfaff, and Seibert (4) determined the carbohydrate content of normal plasma protein fractions isolated by the low temperature ethanolic procedure of Cohn *et al.* (1) and found considerable carbohydrate to be associated with all globulin fractions. It would be of interest to determine which of the serum protein fractions are responsible for the elevation of serum polysaccharide in malignancy and in other conditions. A study of the application to this problem of the fractionation of serum proteins by sodium sulfate and ammonium sulfate has been made and will be published elsewhere; elevation of polysaccharide associated with the albumin fraction in normal pregnancy has been reported (8). It is the purpose of this paper to describe similar studies on patients with carcinoma, arthritis, and infections.

EXPERIMENTAL

Serum was fractionated into albumin, albumin $+\alpha$-globulin, and β-$+\gamma$-globulins by a modified method of Milne (3) with 21.6 per cent and 26.8 per cent sodium sulfate. The γ-globulin was precipitated from serum by the method of Jager and Nickerson (2) using 33 per cent saturated ammonium sulfate. Nonglucosamine polysaccharide (referred to hereafter in this paper simply as "polysaccharide") was determined by the tryptophan method, as previously described (6), on the albumin and the albumin $+\alpha$-globulin fractions after precipitation with absolute ethanol. The polysaccharide associated with α-globulin was estimated by the difference between these two determinations. The γ-globulin polysaccharide was determined after dissolving the precipitate from the ammonium sulfate precipitation in 0.9 per cent saline and reprecipitating with absolute ethanol. Estimation of the polysaccharide associated with β-globulin was achieved by subtracting from the total polysaccharide the sum of the polysaccharide associated with albumin, α-globulin, and γ-globulin.

Protein was determined in all fractions by the biuret reaction. The polysaccharide content of each fraction was expressed as a ratio by dividing the polysaccharide by the protein of the fraction in question. For example, albumin polysaccharide $\div$ albumin $=$ albumin polysaccharide content.

Patients for study were selected from those admitted to the University hospitals. For the studies on carcinoma, most of the samples were from patients with a tentative diagnosis of malignancy before biopsy samples were taken and before any treatment was initiated. Final diagnosis was established in most cases by biopsy.

RESULTS

A summary of the polysaccharide content of the different serum fractions for 48 patients with carcinoma, 15 with benign tumor, 9 with arthritis, 8 with viral infection, and 9 with bacterial infection, and for 17 normal adults is given in Table 1. The carcinoma group included patients with carcinomas of the skin, 4; lung, 11; stomach, 6; pancreas, 1; rectum, 6; kidney, 1; liver, 1; breast, 5; cervix, 5; prostate, 1, and penis, 1. The group of arthritis patients included 6 cases of rheumatoid, 2 of osteo, and 1 of gouty arthritis. The group with viral infections consisted of 5 cases of poliomyelitis in the convalescent stage; 2 of infectious hepatitis; and 1 of venereal lymphogranuloma. The patients with bacterial infections included 4 with tuberculosis; 1 with bronchopneumonia; 1 with osteomyelitis; 1 with tularemia; 1 with brucellosis; and 1 with an infection of the elbow. These data were compared statistically with those for normal adults by the conventional t test.

The average polysaccharide content of the albumin fraction of carcinoma patients was signifi-

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cantly higher than that of normals. A smaller but significant elevation occurred in the benign tumor, arthritis, and bacterial infection groups. However, the average albumin polysaccharide for the carcinoma group was significantly higher than the average for all of the nonmalignant pathologies (t value = 5.6), or that for any single group.

method employed. This may indicate that the fractionation method used gives results somewhat different from electrophoresis as employed by Seibert *et al.* It should be noted that the results of electrophoretic analyses of normal sera by Milne (3) are somewhat different from those of Seibert *et al.*

TABLE 1

SUMMARY OF THE PERCENTILE SERUM POLYSACCHARIDE CONTENT OF SERUM PROTEIN FRACTIONS

Group	No. of cases	Albumin polysaccharide ×100 ÷Albumin			α-Globulin polysaccharide ×100 ÷α-Globulin			β-Globulin polysaccharide ×100 ÷β-Globulin			γ-Globulin polysaccharide ×100 ÷γ-Globulin		
		Average	Range	CV*	Average	Range	CV*	Average	Range	CV*	Average	Range	CV*
Normal adults	17	0.61	0.45–0.78	0.15	2.93	2.28–4.00	0.18	5.65	3.86– 7.46	0.23	2.29	1.92–2.63	0.12
Carcinoma	48	1.70†	0.53–5.42	0.58	4.42†	2.20–8.49	0.37	6.31‡	3.19– 9.07	0.64	2.48‡	1.77–3.58	0.22
Benign tumors	15	0.88†	0.56–1.18	0.35	2.94	2.00–4.41	0.21	5.53	4.00– 6.40	0.29	2.53	2.08–3.34	0.18
Arthritis	9	1.17	0.90–1.58	0.20	4.24†	3.16–4.71	0.20	7.18†	4.73–10.13	0.26	2.15	1.92–2.63	0.10
Viral infections	8	0.70	0.53–1.04	0.23	2.81	1.98–3.48	0.11	7.42†	4.26–10.20	0.25	2.38	2.19–2.50	0.15
Bacterial infections	9	0.86†	0.56–1.26	0.24	3.37	2.46–4.72	0.24	6.90†	5.50– 8.92	0.29	2.16	1.86–2.53	0.11

* Coefficient of variation.
† Significantly higher than the normal value at the 1 per cent level.
‡ 47, rather than 48, cases.

TABLE 2

SUMMARY OF THE PROTEIN DISTRIBUTION AMONG THE SERUM FRACTIONS*

	Albumin	α-Globulin	β-Globulin	γ-Globulin
Normal adults	62	11	13	14
Carcinoma	55	12	15	18
Benign tumors	60	11	14	15
Arthritis	54	11	14	21
Viral infections	59	12	14	15
Bacterial infections	58	12	13	17

* Expressed as per cent of the total serum protein.

The polysaccharide content of the α-globulin fraction was elevated significantly both in patients with carcinoma and in those with arthritis; it was slightly, but not significantly, elevated in patients with bacterial infections. The polysaccharide content of the β-globulin fraction was elevated significantly in patients with arthritis and in those with viral or bacterial infections. The ratio of polysaccharide to protein for the γ-globulin fraction was not influenced by any condition studied.

The distribution of total serum protein among the different fractions is summarized in Table 2. The normal values reported are in fair agreement with those given by Milne (3) and by Jager and Nickerson (2). Results for patients with benign tumors were essentially the same as those for normal adults. All other groups exhibited a decrease in albumin and an increase in one or more of the globulin fractions. In contrast to the large increase of α-globulin in carcinoma sera reported by Seibert *et al.* (5), only a slight increase was noted by the

DISCUSSION

It appears that elevation of total serum polysaccharide in pathological conditions is related to two factors. First, a carbohydrate-rich fraction or fractions may increase with respect to a carbohydrate-poor fraction—as, for example, an increase of any globulin fraction with respect to albumin. Second, the polysaccharide content of a particular protein fraction may increase. It is obvious that the first factor operated in all pathological conditions reported in this study with the exception of the benign tumor group; the second factor was also present in all pathologies; however, the particular protein fractions in which elevation of polysaccharide occurred differed with the condition. In patients with benign tumors, the polysaccharide content of the albumin fraction was slightly but significantly elevated. The polysaccharide content of the albumin fraction was increased greatly in carcinoma and that of the α-globulin was also elevated to a smaller degree, perhaps through increase of the carbohydrate-rich α_2-globulin relative to α_1-globulin. However, the most striking alteration in the sera of carcinoma patients is the increase in albumin polysaccharide. It is this elevation which apparently accounts for the inability of Seibert, Pfaff, and Seibert (4) to calculate the polysaccharide content of sera from carcinoma patients from electrophoretic analysis and the polysaccharide content of isolated normal human serum fractions. Related to this phenome-

non is the finding of Winzler and Smyth (9) with regard to mucoprotein or seromucoid in sera of cancer patients. The albumin fraction, as prepared in our study, should include this seromucoid fraction. In order to determine how much of the elevation of polysaccharide in the albumin fraction is due to mucoprotein, a number of sera from cancer patients were subjected to concurrent albumin polysaccharide and seromucoid determinations. Seromucoid was isolated by the method of Winzler and Smyth (9), with the use of phosphotungstic acid to precipitate the seromucoid after precipitation of other serum proteins by perchloric acid. The carbohydrate moiety was determined by the tryptophan method. A summary of the results is presented in Table 3. Apparently, mucoprotein accounts for some of the elevation of the polysaccharide in the albumin fraction, but, after deducting the mucoprotein polysaccharide, the polysaccharide content of the albumin fraction is still much higher than that in normal sera.

TABLE 3

RELATIONSHIP OF SEROMUCOID POLYSACCHARIDE TO THE POLYSACCHARIDE OF THE ALBUMIN FRACTION*

		MG. PER CENT OF NONGLUCOSAMINE POLYSACCHARIDE ASSOCIATED WITH		CORRECTED ALBUMIN POLYSACCHARIDE†	
GROUP	NO.	Albumin	Seromucoid	Mg. per cent	Per cent of albumin‡
Carcinoma	20	49(32–80)	21(8–43)	28(19–54)	1.09(0.60–2.06)
Normal adults	10	28(22–32)	12(8–18)	16(11–24)	0.40(0.28–0.55)

* Figures in parentheses represent range of values.
† Albumin polysaccharide less seromucoid polysaccharide.
‡ Albumin protein corrected by subtracting the protein of the seromucoid fraction.

Arthritis is characterized by increases in the polysaccharide content of the albumin, α-globulin, and β-globulin fractions, and a noteworthy elevation of γ-globulin protein resulting in an increase of total γ-globulin polysaccharide. The sera of patients with infections exhibited elevations of polysaccharide content in the β-globulin fraction, with only slight elevations in the albumin fraction.

SUMMARY AND CONCLUSIONS

A study has been made of the distribution of nonglucosamine polysaccharide in the serum protein fractions of sera from patients with carcinoma, benign tumors, arthritis, and infections. A method employing fractionation of the serum proteins with sodium sulfate and ammonium sulfate was used. Sera of carcinoma patients exhibited a greatly elevated content of polysaccharide in the albumin fraction in 45 of 48 cases. Only part of this increase was due to polysaccharide in the mucoprotein fraction. The polysaccharide associated with albumin showed smaller elevations in sera of patients with arthritis, benign tumors, and bacterial infections. The α-globulin polysaccharide was elevated significantly in carcinoma and in arthritis. Polysaccharide of the β-globulin fraction was elevated significantly in arthritis and in viral and bacterial infections. No alterations were noted in the polysaccharide content of the γ-globulin fraction.

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Attempts To Produce Gastric Carcinoma Experimentally in a Gastric Ulcer*

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INTRODUCTION

The marked resistance of the glandular stomach of animals to spontaneously occurring cancer is well known. A similar resistance to the experimental production of cancer has been observed, since carcinogenic agents have been fed to mice and rats without producing cancer of the glandular stomach (6). However, when threads saturated with methylcholanthrene were imbedded in the mucosa of the stomach of mice, a cancer developed (6).

In view of these results it has been postulated that the gastric mucus or the superficial gastric mucous cells may serve as a barrier to carcinogens in the diet (4). In order to avoid this possibility, it was decided to produce a chronic ulcer of the glandular stomach of an animal so that the orally administered carcinogen might come into contact with the proliferating cells in the margin of the ulcer (5).

EXPERIMENTAL

Choice of an experimental animal.—The problem of the choice of an animal was first considered. It would have been desirable to have used the dog because of its size, but one of us had found that the pyloric mucosa of the dog has a very high grade resistance to methylcholanthrene, which may be imbedded in or rubbed into the mucosa without causing a tumor within 2 years (4). It was not desirable to use the mouse or rat, because a method for causing a chronic ulcer of the glandular mucosa is not available. The rabbit was not an ideal animal to use, because it is relatively resistant to the formation of neoplasms by the use of carcinogens (3). However, the rabbit was desirable because a chronic ulcer may be produced by excising a piece of pyloric mucosa and by feeding a rough diet (2), or by bilateral vagotomy and by feeding a rough diet. Since a relatively chronic ulcer was a basic necessity for the experiment, it was necesssary to choose the rabbit despite its natural resistance to methylcholanthrene (MCA).

Controls (no operation).—Seven rabbits were given orally 15 mg. of methylcholanthrene (MCA) daily for the first 2 months, in the form of an emulsion in shale oil, each 15 mg. of MCA being dissolved in 0.1 cc. of benzene. After that period the following formula was used: 800 mg. MCA, 5 gm. cetyl alcohol, 1 gm. cholesterol, 6 gm. monoglyceryl stearate dissolved in 80 cc. of shale oil (Texas Co., Altaire Oil, SAE # 1), emulsified in 868 cc. of distilled water, with 50 cc. of 10 per cent Aerosol OT. Five cc. of this emulsion was given orally by tube daily until the end of the experiment.

Four animals were fed for slightly more than 9 months, one for 6 months, and two for slightly over 6 months. At the end of this time no lesions were found other than a very decided atrophic gastritis of the pyloric mucosa in the animal examined at 6 months.

Excision ulcers.—An operation was performed on fourteen rabbits, and an excision ulcer was produced after the method of Beazell and Ivy (1), after which they were given 15 mg. of MCA dissolved in 0.1 cc. benzene (placed in capsules) daily, except Sunday.

Nine rabbits were examined after 1 year and five after 18 months. No abnormality was found other than ulcer scars. One animal was fed MCA for 3.3 years (15 mg. in 0.1 cc. benzene in capsules). On examination, only papillomata were found at the site of a previous ulcer, as described by Beazell and Ivy (1) and Ivy and Cooke (5). Microscopic study showed no evidence of malignancy.

Bilateral vagotomy.—Fifteen rabbits were subjected to bilateral vagotomy (1). After the operation, 5–10 cc. of the following emulsion was administered daily, except Sunday: 800 mg. of MCA, 5 gm. cetyl alcohol, 1 gm. cholesterol, 1 gm. 4-dimethylaminoazobenzene, and 6 gm. of triglyceryl stearate were dissolved in 80 cc. of warm shale oil; and 5 gm. of Aerosol O.T., dissolved in 50 cc. of distilled water and 2 cc. of 1.0 N NaOH, were added; this mixture was then emulsified in

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868 cc. of distilled water; then 1 gm. of 2-acetylaminofluorene was added to the emulsion during vigorous shaking.

Three of the 15 rabbits survived 3 months, five for 4 months, four for 5 months, and three for 8 months. The animals were examined at autopsy, and a section of the liver was removed and examined microscopically. No evidence of malignancy was found, and the liver was normal—one animal dying of pyloric obstruction due to a penetrating pyloric ulcer.

DISCUSSION

The observations on the excision ulcer represent an extension of those reported by Ivy and Cooke (5). In their report, cystic changes were observed in three rabbits which had received MCA for 5–7 months, and epithelial "inclusions" were noted in the region of the papillomatous scar of a chronic ulcer in eight rabbits examined from 3 to 5.5 months after the ulcer was produced.

Unfortunately, the rabbits did not withstand bilateral vagotomy well, as only three survived up to 8 months.

Our failure to observe the development of a cancer in rabbits with a chronic gastric ulcer may be due to the fact that only one of our rabbits lived longer than 18 months. It may also be due to the possibility that, even when the "mucous barrier" is lacking, the MCA is not carcinogenic for the gastric epithelial cells of the rabbit. This latter point will have to be determined by implanting threads impregnated with MCA in the gastric mucosa of rabbits. It is of interest to indicate that neoplasms of the small intestine, which were obtained in mice by feeding MCA (6), were not seen in our rabbits.

The initial objective of this investigation was to obtain evidence concerning the origin, in man, of cancer in a chronic peptic ulcer of the stomach—which is a moot question and may not be answered with finality by clinical observation alone. The negative evidence obtained, to date, in the dog (4) and the rabbit is not conclusive. However, research should perhaps be directed to other species of laboratory animals or to the use of other carcinogens.

SUMMARY

A chronic gastric ulcer was produced in rabbits by excision or by bilateral vagotomy, and then methycholanthrene was fed for periods ranging from 3 to 18 months (one for 3.3 years and 30 for from 8 to 18 months) with the idea that a cancer might occur in the ulcer. A cancer was not observed, although cystic changes in and atrophic gastritis of the gastric mucosa and epithelial inclusions in papillomatous scars of healed ulcers were observed.

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The Effect of Xanthopterin and Related Agents on the Proliferation of Rabbit Marrow Cells *in Vitro**

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The experimental chemotherapy of neoplastic disease could be placed on a rational basis by the discovery of consistent metabolic differences between normal and malignant cells. Specifically, differences in nutritional requirements might be used to effect inhibitions of neoplastic growth while the normal tissues might be left unharmed. It is therefore a matter of considerable interest when the discovery of clear distinctions between normal and malignant cells, with respect to their reactions to metabolites and antimetabolites, is reported.

Claims of differential growth responses of normal and malignant cells to xanthopterin and related substances in suspension cultures have been made in a series of recent publications (3–11). Xanthopterin at an optimal concentration of 5 μg. per milliliter was reported to cause a significant increase in the proliferation of cells from bone marrow (3) and from other normal tissues (6). Several other pteridines were found to have a less pronounced effect than xanthopterin (4). Xanthopterin-7-carboxylic acid and 2-amino-4-hydroxy-7-methyl-pteridine were said to act as anti-xanthopterins and to inhibit marrow cell proliferation in cultures (4). Similarly, normal blood serum was found to accelerate marrow cell proliferation, while blood sera from patients with pernicious anemia or with various neoplastic diseases inhibited normal cell proliferation (5). On the other hand, neoplastic cell proliferation in culture was reported to be increased by sera from patients with neoplastic disease and to be inhibited by normal serum, while anti-xanthopterins acted as stimulants for neoplastic cell proliferation and xanthopterin acted as an inhibitor (7). In experiments with human bone marrow cultures, the results were found to correspond to those obtained with animal marrow (8). It was reported that normal human urine, as well as urine from patients with malignant tumors, contained substances that accelerated the rate of proliferation in bone marrow cultures (11). One such material isolated from human urine, a so-called "vitamin B_{14}," was said to be active in very small amounts, both in promoting the increase of bone marrow cells and in depressing increase in Brown-Pearce rabbit tumor cells *in vitro* (9). "Vitamin B_{14}" was rivaled in activity by the products of incubation of xanthopterin or folic acid with xanthine oxidase preparations (10).

These reports tended to draw such sharp distinctions between normal and neoplastic cell growth that attempts to confirm them became imperative. The present paper describes investigations dealing with salient claims of the cited authors and advances reasons for concluding that some of their statements might be reconsidered.

MATERIALS AND METHODS

The procedure followed was that described by Norris and Majnarich (3), or adaptations of it, with supplements of agents added to suspensions of rabbit femoral marrow. Aseptic technic was used throughout.

In most instances, the following series of operations was carried out. A young, adult, male domestic rabbit was deprived of food for 1 day. It was then bled from the heart and killed by a blow on the head. The blood was incubated at 37° C. for 15 minutes to allow it to clot: serum was then removed for cutures of the Osgood-Brownlee type (12). The femurs were removed, swabbed with alcohol, and the heads of the femurs sawed off to permit insertion of the shaft into a sterilized rubber tube attached to a 30-ml. syringe. The marrow was then expressed into a sterile 50-ml. centrifuge tube bearing the suspension fluid. This fluid was either Tyrode's solution without glucose or Gey's balanced salt solution without glucose (both for the Norris casein hydrolysate medium): or the Osgood-Brownlee type medium of 35 per cent autologous or homologous serum and 65 per cent Gey's balanced salt solution with glucose. The contents of one femur were suspended in

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10 ml. of the suspension fluid by gently stirring and shaking. Thereafter, the suspension was either strained through gauze or centrifuged at 500 r.p.m. for 1 minute to remove the gross clumps of tissue. After centrifugation, the central portion of the suspension was pipetted off and used for the cultures, after dilution with more of the same suspension medium if necessary to establish a desired cellular concentration. The suspensions used contained initially about 2,000 to 20,000 nucleated cells per cubic millimeter, and the number of erythrocytes averaged about 9 times the number of nucleated cells.

For one culture, 3.0 ml. of this original cell suspension was taken, and supplements were added. For the Norris casein hydrolysate medium, the supplements were 0.3 ml. of neutralized casein acid hydrolysate,[1] 0.015 ml. of 1 per cent 1(−)tryptophan (General Biochemicals, control No. 17359), and 0.3 ml. of distilled water containing the tested agent, if any. For the Osgood-Brownlee type medium, the supplement was 0.3 ml. of distilled water, which contained the tested agent in the case of experimental cultures. With either casein hydrolysate or serum medium, 2.5 ml. of this mixture was placed in the culture vial, and the rest was used for zero-time cell counts and for stained smears.

The culture vials were 5-ml. Pyrex glass vials. Each contained one glass bead for stirring. The vials were closed with rubber caps. They were placed in a rack and shaken in a Warburg bath at 37° C. for the duration of the experiment, which was usually 5 or 6 hours.

Cell counts of erythrocytes and of nucleated cells were made with counting chambers, in the usual manner, at 0, 3, and 5 or 6 hours' incubation. For this purpose, 0.3 ml. was removed from each 2.5-ml. culture at 3 hours by means of a sterile needle inserted through the rubber cap. Concurrently smears or drops of the suspensions were air-dried on microscope slides, stained with Wright's stain, or fixed in absolute methyl alcohol and stained with Jenner-Giemsa for counts of mitotic frequency in different cell types.

After three preliminary experiments, seven experiments were performed with xanthopterin at various concentrations in both casein and serum media, one experiment was carried out with 2-amino-4-hydroxy-7-methyl pteridine, one with 7-methyl xanthopterin, two with vitamin B_{12}, one with normal human urine, three with normal and leukemic human sera, and two with folic acid and xanthopterin incubated with liver homogenates.

We are indebted to Merck and Company for one sample of xanthopterin. Another sample of xanthopterin that came from the same lot as that employed by Norris and Majnarich was kindly supplied by Dr. George H. Hitchings of the Wellcome Research Laboratories, as was the 7-methyl xanthopterin. We are indebted to Dr. C. K. Cain of the Cornell University chemistry department for the 2-amino-4-hydroxy-7-methyl pteridine. We are obligated to Dr. Karl Folkers of Merck and Company for a gift of vitamin B_{12} in solution at 50 μg/ml.

The normal human urine was sterilized by filtration before use.

Normal human serum was from one of the authors (J. J. B.), and leukemic human sera were kindly provided by Dr. David A. Karnofsky of this Institute from patients at Memorial Hospital.

The first patient was an adult male with lymphatic leukemia; his white blood cell count was about 1,000,000/c mm, and he was under treatment with adrenocorticotropic hormone. The second patient was also an adult male with lymphatic leukemia; he had received one dose of 5 and another of 15 mg. A-methopterin. The third patient had not been treated; she was a 56-year-old woman with subacute myelogenous leukemia, her peripheral white blood cell count being 20,000/c mm.

Liver homogenate was prepared for incubation with folic acid or xanthopterin as follows: 7.7 gm. of liver from an adult male albino rat was ground under sterile conditions in a glass homogenizer with 5 ml. of Gey's solution containing 25 μg. streptomycin and 25 units penicillin per milliliter. The amount of Gey's solution was then increased by 10 ml. To 6.0 ml. of the liver homogenate was added 0.2 ml. of Gey's solution with glucose containing xanthopterin in sufficient quantity to give a final concentration of 50 μg. of added xanthopterin per milliliter of liver homogenate. The same was done with pteroylglutamic acid. Similar preparations of rabbit liver homogenate with xanthopterin and with folic acid were made. Liver homogenate without supplement, homogenate with PGA, and homogenate with xanthopterin were incubated at 37° C. for 43 hours for rat liver and for 45 hours for rabbit liver. Sterility tests with Brewer's thioglycollate medium indicated absence of bacteria from all preparations. Rabbit marrow cell suspensions were supplemented with 0.1 ml. of incubated liver homogenate per milliliter of suspension; each experimental culture then

[1]From 2.0 ml. of General Biochemicals' "vitamin-free" 10 per cent casein hydrolysate for microbiological procedures, control No. 17810, brought to neutrality with 0.06 ml. of 5 N NaOH.

TABLE 1

Mitotic Phase Counts in Stained Smears of Marrow Cultures

Supplement	Medium	Nucleated cells counted	Number of cells in mitotic phases: Erythroid Pro	Erythroid Meta	Erythroid Ana	Erythroid Telo	Other Pro	Other Meta	Other Ana	Other Telo
Xanthopterin	Casein	5,800	0	3	0	1	0	1	0	1
Controls	Casein	2,200	0	4	0	1	0	5	0	2
Xanthopterin	Serum	2,200	2	10	2	5	3	8	1	6
Controls	Serum	1,000	5	2	1	3	0	1	0	0
Vitamin B_{12}	Serum	6,000	2	24	1	14	3	12	3	7
Controls	Serum	1,200	0	3	1	2	0	0	0	0
Urine	Serum	3,000	32	15	0	16	0	1	0	0
Controls	Serum	3,000	11	25	3	13	4	4	1	0
Leuk. serum	Serum	15,500	72	61	2	18	20	34	5	16
Norm. serum	Serum	13,500	37	18	1	3	8	4	0	5
Liver homog. & xanthopt.	Serum	4,500	3	6	1	10	3	17	4	1
Liver homog. & folic acid	Serum	4,500	1	14	4	8	1	7	2	3
Liver homog. alone	Serum	4,500	1	10	1	1	6	14	2	1

TABLE 2

Effect of Xanthopterin on Mitotic Rates in Rabbit Bone Marrow Suspensions in Autologous Serum Medium

Each entry is based on analysis of 200 nucleated cells

Xanthopterin (μg/ml)	Percentage of mitoses: Erythroid 0 hr.	Erythroid 3 hr.	Erythroid 6 hr.	Other 0 hr.	Other 3 hr.	Other 6 hr.	Total 6 hr.
None (1)	4.0	0	1.0	0	0.5	0	1.0
None (2)		0.5	0		0	0	0
5 (1)	1.0	1.5	1.0	0	0	2.0	3.0*
5(2)		0.5	2.5		1.5	0.5	3.0*
10	1.0	0	0	0.5	1.0	2.0	2.0†
20	0	1.0	1.5	0.5	0.5	0.5	2.0†

* Together, significantly different from controls at 6 hours by "t" test.
† Together, not significantly different from controls at 6 hours by "t" test.

TABLE 3

Effect of Vitamin B_{12} on Mitotic Rates in Rabbit Bone Marrow Suspensions in Autologous Serum Medium

Each entry is based on analysis of 200 nucleated cells

Vitamin B_{12} (μg/ml)	Percentage of mitoses: Erythroid 0 hr.	Erythroid 3 hr.	Erythroid 6 hr.	Other 0 hr.	Other 3 hr.	Other 6 hr.	Total 6 hr.
First experiment:							
None	1.0	0.5	0.5	0	0	0	0.5
5×10^{-4}	1.0	0	0	0	1.5	0	0
5×10^{-3}	0	0.5	0	1.0	0	0.5	0.5
5×10^{-2}	2.0	1.0	2.0	0	0.5	0	2.0*
5×10^{-1}	1.0	1.0	1.5	1.0	1.0	0.5	2.0*
5	0	0	0.5	0	0.5	2.5	3.0*
Second experiment:							
None	0.5	0	0.5	0	0	0	0.5
5×10^{-7}	0.5	0	1.5	1.0	0	0	1.5
5×10^{-6}	0.5	2.0	0.5	0	0	0	0.5
5×10^{-5}	0	0.5	0	0	0	0	0
5×10^{-4}	1.0	1.5	1.5	0.5	0.5	1.5	3.0
10	0	0	1.5	0	0	0	0.5

*Together, significantly different from controls at 6 hours.

contained the equivalent of 5 μg. of added xanthopterin or folic acid per milliliter. Control cultures were supplemented with liver homogenate alone.

Results of cell counts under different conditions were treated statistically, the "t" test being used to assess the significance of differences between means. Mitotic counts also underwent statistical analysis.

RESULTS

The poor condition of cells in the casein hydrolysate medium led to greater use of serum medium by the authors. Higher mitotic rates in serum medium are indicated by data of Table 1, which presents a summation of the counts of mitotic phases in several experimental groups. It is apparent from this table that mitosis was not halted at any particular stage by the supplements used.

Mitoses counted in three experiments with xanthopterin in casein medium were so low in frequency as to render the analysis of the effect of xanthopterin concentration fruitless. Results of the experiment with xanthopterin in serum medium are presented according to time and xanthopterin concentration in Table 2. Data for other relatively high mitotic rate experiments are given in Tables 3, 4, 5, and 6.

Certain points are to be noted in the accompanying tables. A significantly higher mitotic frequency occurred with 5 μg. of xanthopterin per milliliter in serum medium than in controls. This did not hold for cultures in casein medium. Vitamin B_{12} at 0.05 to 5 μg/ml appeared to stimulate or maintain mitosis to some extent over the 6-hour incubation period. Supplementation of cultures with 0.1 ml. of normal human urine did not cause statistically significant increases in mitotic frequency. Experiments with sera from three leukemic patients showed higher mitotic rates in cultures supplemented with the leukemic sera than in those supplemented with normal human serum; only in one experiment with serum from a leukemic patient under ACTH treatment was the increase statistically significant on the 95 per cent level of confidence. Supplementation of marrow cultures with an incubated mixture of xanthopterin or folic acid with liver homogenate did not produce significant increases in mitotic rate over cultures supplemented with liver homogenate alone.

The results of counts of erythrocytes and nucleated cells after 5 or 6 hours' incubation are presented in Table 7 as percentage changes, with standard deviations, from counts made at 0 hours' incubation.

The statistical significance of the differences between mean changes in cell counts over the incubation period under various treatments was determined by means of the "t" test. For this test, it will be recalled, the value of the difference divided by its standard deviation must be assessed.

In few of the reasonable comparisons could the difference between two mean percentage changes be regarded as statistically significant on the 95 per cent confidence level. In none of the comparisons was there a significant difference between the changes of nucleated cell numbers during incubation. Changes in erythrocyte numbers were significantly different in only three comparisons. These were (*a*) the increase in erythrocytes in casein cultures given 5 μg. of xanthopterin per milliliter, as compared with control casein cultures; (*b*) the increase in erythrocytes in cultures with 5 μg. of xanthopterin per milliliter in both media, as compared with cultures receiving 10 μg. of xanthopterin per milliliter; and (*c*) the increase in erythrocytes in cultures supplemented with 5 μg. of 2-amino-4-hydroxy-7-methyl pteridine, as compared with the decrease in control cultures in casein medium. Statistical significance on the 95 per cent level was nearly attained by the difference between mean percentage erythrocyte increases in cultures receiving incubated liver homogenate alone and in those supplemented with xanthopterin and liver homogenate mixture.

The following comparisons showed no statistically significant differences between changes either for nucleated cells or for erythrocytes: xanthopterin at 5 μg/ml in serum medium versus controls in serum; the same in both media together; xanthopterin at 10 or 20 μg/ml in either casein medium or both media together versus the appropriate controls; xanthopterin at 5 μg/ml or at 10 μg/ml in both media versus 20 μg/ml xanthopterin; 7-methyl xanthopterin at 5 μg/ml in casein medium versus casein controls; 7-methyl xanthopterin at 5 μg/ml in casein medium versus xanthopterin at the same concentration; 2-amino-4-hydroxy-7-methyl pteridine at 5 μg/ml in casein medium versus xanthopterin at 5 μg/ml in casein medium; vitamin B_{12} at all concentrations versus controls in serum medium; normal urine in serum medium versus serum controls; leukemic human sera versus normal human serum; and liver homogenates incubated with xanthopterin or folic acid versus liver homogenates incubated alone.

DISCUSSION

The results of the foregoing experiments differed in many respects from the published results of Norris and Majnarich. This was true of experiments employing the medium preferred by Norris

TABLE 4

Effect on Mitotic Rates of Normal Human Urine, 0.1 ml/ml rabbit Bone Marrow Suspension in Autologous Serum Medium

Each entry is based on analysis of 200 nucleated cells at 0 hours, 400 cells at 3 and at 6 hours

	Percentage of mitoses						
	Erythroid			Other			Total
	0 hr.	3 hr.	6 hr.	0 hr.	3 hr.	6 hr.	6 hr.
Control 1	2.0	2.25	1.0	2.0	0	0	1.0
Control 2	4.5	3.25	0.25	1.0	0	0	0.25
Control 3	0.5	1.5	1.25	1.0	0	0.25	1.5
Urine 1	4.0	1.5	1.75	0	0	0	1.75*
Urine 2	5.0	1.75	3.0	0	0	0	3.0*
Urine 3	2.0	1.75	0.5	0.5	0	0	0.5*

* Not significantly different from controls at 6 hours by "t" test.

TABLE 5

Effect of Normal and Leukemic Human Sera on Mitotic Rates in Rabbit Bone Marrow Suspensions in Autologous Serum Medium

Each entry is based on analysis of 1,000 nucleated cells

Serum	Percentage of mitoses						
	Erythroid			Other			Total
First experiment: 1st patient (ACTH)	0 hr.	3 hr.	6 hr.	0 hr.	3 hr.	6 hr.	6 hr.
Normal	1.7	0.1	0.2	0.2	0	0.6	0.8
" (dupl.)	1.3	1.0	0.5	0.3	0.1	0.3	0.8
Leukemic	3.2	1.8	1.6	0.3	0.9	0.9	2.5*
" (dupl.)	2.8	1.2	1.2	0.5	1.0	1.2	2.4*
Second experiment: 1st patient (ACTH)	0 hr.	3 hr.	6 hr.	0 hr.	3 hr.	6 hr.	6 hr.
Normal	0.4	0.1	0.1	0	0	0	0.1
" (dupl.)	0	0	0.3	0	0	0	0.3
Leukemic	0.2	0.4	0.8	0.1	0.2	0.5	1.3†
" (dupl.)	0.4	0	0.3	0.2	0	0.1	0.4†
Third experiment: 2d patient (A-methopterin), 3d patient (untreated)	0 hr.		5 hr.	0 hr.		5 hr.	5 hr.
Normal	0		0.2	0		0.2	0.4
" (dupl.)			0.2			0.2	0.4
2d leukemic	0		1.6	0		0.4	2.0‡
" " (dupl.)			0.2			0.8	1.0‡
3d leukemic	0.4		0.6	0.4		1.0	1.6‡
" " (dupl.)			0.2			0.6	0.8‡

* Significantly different from controls at 6 hours by "t" test.

† Not significantly different from controls at 6 hours by "t" test.

‡ Not significantly different from controls at 6 hours by "t" test individually or collectively.

TABLE 6

Effect of Xanthopterin or Folic Acid Incubated with Liver Homogenate on Mitotic Rates in Rabbit Bone Marrow Suspensions in Autologous Serum Medium

Each entry is based on analysis of 1,000 nucleated cells for rat liver at 0 hours, 2,000 for rat liver at 5 hours, 500 for rabbit liver at 0 hours, 1,000 for rabbit liver at 5 hours

Supplement	Percentage of mitoses				
	Erythroid		Other		Total
	0 hr.	5 hr.	0 hr.	5 hr.	5 hr.
Rat liver homogenate incubated					
With xanthopterin	0.5	0.65	0.7	0.75	1.4*
With folic acid	0.4	0.65	0.4	0.35	1.0*
Alone	0.6	0.2	0.5	0.65	0.85
Rabbit liver homogenate incubated					
With xanthopterin	0.4	0	0.6	0.1	0.1*
With folic acid	1.6	0.1	0.2	0.1	0.2*
Alone	0.4	0.1	0.6	0.3	0.4

* Collectively, not significantly, different from supplements of liver homogenate alone at 5 hours.

and Majnarich (4), which was Tyrode's solution without glucose plus casein hydrolysate and tryptophan, as well as of experiments in which a serum medium was used. The latter was our medium of preference for the following reasons: (*a*) with the Norris casein medium, we observed extreme crenation of erythrocytes and considerable damage to nucleated cells, very few of which became mitotic; (*b*) Norris and Majnarich (3) reported xanthopterin effects on cell proliferation in the Osgood-Brownlee (12) serum medium without glucose equivalent to the xanthopterin effects in casein medium; and (*c*) Hays (1) had earlier reported that liver extract or normal serum increased erythrocyte maturation in marrow cultures in Tyrode's solution, whether or not glucose and/or casein hydrolysate had been added. We therefore used autologous or homologous rabbit serum plus Gey's salt solution with glucose in many of our experiments. In this medium the cells maintained a better appearance and carried out apparently normal mitoses over the 6 hours of incubation. Cell counts in the serum medium were still somewhat doubtful, however, because of the occasional occurrence of small clumps involving both nucleated cells and erythrocytes.

An interesting feature of our results was the lack of correspondence between nucleated cells and erythrocytes with respect to their changes in frequency. In contrast, Norris and Majnarich usually (e.g., 3, 5, 10) reported changes in counts in the same direction for both erythrocytes and nucleated cells. Plum (13), however, found that marrow cultures supplemented with liver extract showed decreases in numbers of nucleated cells, while non-nucleated cells increased in number. A majority of our experiments showed similar tendencies.

Results with xanthopterin were far less striking than those reported by Norris and Majnarich (3, 4). For instance, the percentage of increases in cell counts found with the reportedly optimal supplementation of 5 μg of xanthopterin per milliliter of cell suspension were far below the published increases of about 100 per cent (3, 4). The changes in cell counts in our cultures supplemented with 5 μg. of xanthopterin per milliliter were not significantly different from those in unsupplemented cultures for nucleated cells in either medium or for erythrocytes in serum medium. The increase of erythrocytes in our 5-μg. xanthopterin cultures in casein medium was significant on the 95 per cent level, however, with respect not only to controls but also to cultures supplemented with 10 μg. of xanthopterin per milliliter.

The increase of erythrocytes in the cultures supplemented with 2-amino-4-hydroxy-7-methyl pteridine stands in opposition to the claim of Norris and Majnarich (4) that this agent inhibits the proliferation of normal cells. Nor does the absence of significant differences between the effect

TABLE 7

MEAN AND STANDARD DEVIATION OF PERCENTAGE CHANGES IN CELL COUNTS FROM 0 TO 5 OR 6 HOURS

TREATMENT	MEDIUM	NUMBER OF CULTURES	PER CENT CHANGE IN CELL COUNTS: Nucleated cells, Mean	Nucleated cells, St. dev.	Erythrocytes, Mean	Erythrocytes, St. dev.
Controls through B_{12} expt.	Casein	11	−10.4	± 4.04	− 0.9	± 8.12
Controls through B_{12} expt.	Serum	6	− 8.6	8.74	31.1	18.8
All controls above	Both	17	− 9.7	3.82	9.8	7.22
Xanthopterin 2.5 μg/ml	Casein	1	3.5		17.8	
Xanthopterin 5 μg/ml	Casein	7	−11.3	8.44	13.6	5.12
Xanthopterin 5 μg/ml	Serum	4	14.3	6.23	16.4	17.7
All xanthopterin 5 μg/ml	Both	11	− 2.0	6.86	14.7	6.64
Xanthopterin 7.5 μg/ml	Casein	1	−15.4		−17.7	
Xanthopterin 10 μg/ml	Casein	4	−15.7	7.87	− 7.4	7.05
Xanthopterin 10 μg/ml	Serum	1	10.0		−17.0	
All xanthopterin 10 μg/ml	Both	5	−10.5	6.74	− 9.3	5.79
Xanthopterin 20 μg/ml	Casein	3	− 1.0	12.9	−11.1	12.2
Xanthopterin 20 μg/ml	Serum	1	11.1		−10.1	
All xanthopterin 20 μg/ml	Both	4	2.1	9.59	−10.9	8.63
7-Me-xanthopterin 5 μg/ml	Casein	2	−18.7	0.77	3.7	23.7
7-Me-pteridine 5 μg/ml	Casein	2	− 5.8	0.7	17.5	1.6
B_{12}, 5×10^{-7} to 10 μg/ml	Serum	10	−24.4	3.41	0.7	5.96
Controls for urine	Serum	3	−12.1	6.52	31.6	4.63
Human urine 0.1 ml/ml	Serum	3	−20.2	6.21	33.3	11.5
Normal human serum	Serum	8	−36.4	10.8	− 9.9	12.7
Leukemic human sera	Serum	10	−24.0	6.99	−33.7	11.6
Liver homogenates (LH)	Serum	4	− 2.9	6.32	9.4	3.56
LH with xanthopterin	Serum	4	6.5	8.21	51.5	19.5
LH with folic acid	Serum	4	− 4.4	2.67	11.2	5.42

of similar concentrations of xanthopterin, 7-methyl xanthopterin, and 2-amino-4-hydroxy-7-methyl pteridine agree with their report (4).

Normal human urine was not found to produce a significant increase in proliferation of nucleated cells or erythrocytes over control cultures in serum medium, apparently in contradiction to published results (11).

In three experiments with leukemic human sera, they were not found to cause a statistically significant greater decrease in cell count than normal human serum. These results would seem to contradict those of Norris and Majnarich (5, 8, 11).

Nor were our results with an attempted substitute for "vitamin B_{14}" as dramatic as those reported (9, 10). Supplementation of marrow suspension cultures with the products of incubation of xanthopterin with rat and rabbit liver homogenates produced the greatest percentage increases in erythrocytes found, but these were statistically significantly greater than those given by liver homogenate alone only in the case of rat liver and not when both rat and rabbit experiments were considered together. The increases were still below 100 per cent, in contrast to the reported increases of the order of multiples of 100 per cent (10).

With reference to the published increases in nucleated cells of about 1,000 per cent over 7½ hours in cultures supplemented with "vitamin B_{14}" (9), it is to be regretted that Norris and Majnarich did not report any records of permanent microscopic preparations. If data such as those of Lewis (2) are applicable, which indicate mitoses of cultured cells to last about 1 or 2 hours, a tenfold increase in nucleated cells in 7½ hours would require that each nucleated cell initially present pass, on an average, through 3–4 mitotic generations in that time, and perhaps half the cells might be in overt mitosis at any instant. The mitotic interphase would be greatly shortened, and the cytological picture would be spectacular.

In our stained smears, cells in mitosis were never found to exceed a few per cent of the nucleated cells present. Mitotic rates at the end of incubation periods were significantly higher in experimental cultures than in controls in three cases only. For example, cultures in serum medium supplemented with 5 μg. of xanthopterin per milliliter of cell suspension had terminal mitotic rates of 3 per cent, and these same cultures had the greatest mean percentage increase in nucleated cells observed, approximately 14 per cent. Intermediate concentrations of vitamin B_{12}, which is known to be effective in the treatment of Addisonian pernicious anemia (14), also gave terminal mitotic rates of several per cent, but nucleated cell counts decreased during 6 hours of incubation. Cultures supplemented with sera of leukemic human patients had mitotic rates higher than those supplemented with normal serum, but only in one of three experiments was statistical significance attained. The fact that the leukemic serum in this experiment was obtained from a patient being treated with ACTH recognizably complicates interpretation of the results. Despite the occurrence of mitoses, cell counts in individual cultures supplemented with leukemic sera either fell or remained near the initial level.

To recapitulate: the noteworthy finding of this investigation is the uniform lack of congruence between our results and those of Norris and Majnarich. There are reasons for doubt as to the adequacy of the method, considering the poor condition of cells kept in the casein hydrolysate medium. Nor does the serum medium appear to be ideal. Nevertheless, in the light of our work with marrow cells, the conclusions reached by these authors with respect to the effect of xanthopterin, supposed anti-xanthopterins, and the like on the multiplication of normal cells *in vitro* appear doubtful at best. It does not seem likely a priori that the unsatisfactory technical conditions would be improved by use of some other cell suspensions employed by Norris and Majnarich (6) and prepared by grinding liver, kidney, or other organs in a Waring Blendor. Nor does it seem necessary at this point to pursue their claims (7, 9) with respect to the antithesis between normal and malignant cells in their response to xanthopterin, anti-xanthopterins, and "vitamin B_{14}."

SUMMARY

1. The growth of cells in suspension cultures of rabbit femoral bone marrow has been studied in an unsuccessful attempt to confirm the published statements of Norris and Majnarich with respect to the effects of supplements on the proliferation of normal cells in suspension.

2. A medium of the Osgood-Brownlee type composed of serum and Gey's salt solution was found to be preferable to a medium of Tyrode's solution, casein hydrolysate, and tryptophan.

3. There was an inconclusive indication of slightly increased cellular proliferation in cultures supplemented with xanthopterin at 5 μg/ml.

4. Increase in erythrocytes nearly attained statistical significance on the 95 per cent level in cultures supplemented with rat liver homogenate incubated with xanthopterin.

5. Mitotic rates were slightly increased in serum cultures supplemented with vitamin B_{12} or with leukemic human sera.

6. Failure resulted from attempts to confirm the reported inhibitory effects on normal cellular proliferation of 2-amino-4-hydroxy-7-methyl pteridine, the accelerating effects of normal human urine or serum, and the inhibitory effects of leukemic human serum.

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Cytoplasmic Proteins*

A Partial Physical and Chemical Characterization of the Cytoplasmic Proteins

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Sufficient attention has yet to be given to the cytoplasmic proteins in the problem of carcinogenesis. In fact, the mechanism or site of action of the various carcinogens within a normal cell in the process of malignant transformation has, for the most part, remained unknown; and the possibility still exists that carcinogenesis may be an altered protein metabolism. In a recent review of cancer proteins, Toennies (28) has stressed the need for additional information concerning the proteins of normal and malignant tissues. The further examination of these proteins might yield information on the relationship between tissue proteins and those of blood plasma, as well as on the protein differences existing between normal and malignant tissues.

The purpose of the investigations reported here was to establish a procedure for the examination of the soluble tissue proteins which would be rapid, allow a minimum of handling, give uniform results, and permit the isolation of sufficient material for further study. By following the differential centrifugation method of Claude (4), the soluble cytoplasmic proteins can be obtained in the supernate fraction of a tissue homogenate. An adequate characterization of the complex protein mixture found in the supernate requires the parallel application of both physical and chemical methods of analysis. The work has now progressed to a point allowing a preliminary report to be made of the electrophoretic and chemical analyses of two rat fibrosarcoma extracts and of fractions of these extracts prepared by ammonium sulfate precipitation.

Of the two rat fibrosarcomas employed in this study, one was originally induced with benzpyrene in this laboratory, while the other was induced with methylcholanthrene by W. H. Lewis These tumors were chosen because of their rapid growth and the high percentage of positive takes on transfer into rats of the Wistar strain. The growths do not become excessively necrotic, and they have a fairly uniform cell population.

During recent years, electrophoretic analyses of tissue extracts have been made by numerous investigators—for example, by Abrams and Cohen (1); Holmes and Morrison (11); Jacob (12); Luck, Nimino, and Tostado (15); Miller, Green, Kolb, and Miller (18); Roberts and White (21); Sorof and Cohen (23); Stern, Reiner, and Silber (24); Taylor, Green, and Cori (25); White and Dougherty (29); and others.

METHODS

The supply of tumors was maintained by inoculating batches of twenty-five rats with tumor material at 3-week intervals.

Nitrogen analyses were determined by the micro-Kjeldahl procedure, and nonprotein nitrogen was calculated on the material not precipitated by 10 per cent trichloroacetic acid. Protein concentrations were assumed to be the difference between total nitrogen and the nonprotein nitrogen × 6.25. Because of the incomplete separation of the phospholipid moiety attached to the precipitated protein, the values for protein nitrogen concentration are somewhat high. The method of Berenblum and Chain (3) was used in determining phosphorus, while pentose- and desoxypentosenucleic acids were estimated by the orcinol and diphenylamine reactions as modified by Schneider (22). Dry weight was determined by drying to constant weight at 25° C., and pH was measured with the glass electrode. Centrifugal force values were calculated at the centers of the centrifuge tubes.

All electrophoretic experiments were carried out in veronal buffer of pH 8.6 and 0.1 ionic strength at 0.4° C. in the Tiselius apparatus, with the schlieren scanning method of Longsworth (14). Be-

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fore electrophoresis, the protein samples were dialyzed by means of a cellophane sack versus two separate 200-ml. portions of buffer, and finally the sack was suspended for 24 hours in the bulk buffer fluid. The dialyzed protein solutions were clarified, if necessary, by centrifugation. Mobilities and relative compositions were determined by the method of Tiselius and Kabat (27) on patterns of the descending limb.

EXPERIMENTAL

About 18–23 days following inoculation the tumors were removed and stored on ice after all the adhering blood vessels, fat, connective tissue and necrotic parts were eliminated. Two to three hundred gm. of tissue were collected in 3–3.5 hours. Efforts were made to preserve the original state of the tissue by the use of as mild procedures as possible, carried out in the cold room at 4° C. The tumor tissue was mashed by passing 25-gm. portions through a stainless steel masher (1-ml. holes) into a 1-liter beaker containing 100–150 ml. of extraction medium (listed in Table 1). By taking 30-ml. aliquots, the whole mash was homogenized with seven to ten strokes of the glass homogenizer (19). This viscous homogenate was diluted with additional extraction fluid to a tissue concentration of 40–55 per cent by weight and a total volume of 500–650 ml. The total material loss for the combined mash ng and homogenizing operations was 9–10 per cent of the initial wet weight.

The homogenate, after thorough mixing, was centrifuged in three or four 250-ml. centrifuge bottles at 1,000 *g* for 15 minutes. This insured the elimination of most of the unbroken cells, cell nuclei, cellular debris, and some of the mitochondria. After the supernatant fluids were decanted, each residue was re-extracted with a fresh 50- to 80-ml. of medium, centrifuged as before, and the supernates decanted. To remove the remaining large granules and mitochondria, the first and second supernates were centrifuged at 2,000 *g* for 30 minutes, either separately or as a pool, in 50-ml. centrifuge bottles. These resulting supernates were further centrifuged for 2 hours in 50-ml. lusteroid tubes in a refrigerated angle centrifuge at 35,000 *g*. The solution temperature usually rose from 0° to 9° C. during the last centrifugation. On the assumption that the mean diameter of the microsomes is 5×10^{-6} cm., it was calculated[1] that 2 hours was ample for precipitating these particulates. The final supernate had a graded opalescence from the top to the bottom of the tube. The clearest part of the supernate was collected and stored either on ice at 0° C. or in the deep freeze at −25° C.

When the first and second extracts were processed separately, the final volume yields of the first extracts, based on the total volume of extraction medium added, were 40–41 per cent for the sucrose media and 49–54 per cent for the salt

TABLE 1

PER CENT OF THE TOTAL NITROGEN AND PHOSPHORUS EXTRACTED FROM THE TISSUES*

	Methylcholanthrene-induced fibrosarcoma											
	Nitrogen									Phosphorus		
Extract medium	1st extract	2d extract	Total	Protn. 1st	Protn. 2d	Total protn.	NPN 1st	NPN 2d	Total NPN	1st extract	2d extract	Total
Isotonic 0.14 M potassium chloride	36.2±1.2		36.2±1.2	29.0±0.2		29.0±1.4	7.2		7.2	29.0±0.4		29.0±0.4
Isotonic 0.25 M sucrose	36.8±1.1	9.6±0.2	46.4±1.3	30.8±0.1	6.9±0.1	37.7±1.5	6.0	2.7	8.7	29.7±0.5	8.2±0.3	37.9±0.8
0.5 M potassium chloride + 0.03 M sodium bicarbonate, pH 8.0	38.3±0.8	14.4±0.3	52.7±1.1	31.1±0.2	7.8±0.1	38.9±1.4	7.2	6.6	13.8	29.4±0.6	10.0±0.3	39.4±0.9
	Benzpyrene-induced fibrosarcoma											
Isotonic 0.25 M sucrose or Isotonic 0.25 M sucrose +0.03 M potassium bicarbonate, pH 8.0			45.4±0.7			37.4±0.7			8.0			38.5±0.5

* Results are expressed as the mean ± the standard deviation based on 3 or more values.

$$\text{St. dev.} = \sqrt{\sum \frac{d^2}{n-1}}$$

[1] Equation used for calculating velocity of particles (7):

$$V = 2/9\,\frac{\delta - r}{\eta} \cdot a^2(2\pi S)^2 \times R \text{ cm/sec.}$$

Where R = radius of arm in cm.
S = No. of rev/sec.
δ = particle density.
r = medium density.
η = viscosity in poises.
a = radius of particle.

media. The second extracts gave yields, consistent for all media, of 68–74 per cent of the volume used in the re-extraction. On the pooled extracts 50 per cent yields were obtained. These recoveries are low, since only part of the supernates was collected. The total extract volume obtained, in all cases, was between 215 and 375 ml.

All precipitates and residual fluids remaining after each centrifuge operation were added to the original residue fraction. The total time required for preparation of the final extract varied between 13 and 15 hours.

RESULTS

General properties of the tumor homogenate and extracts.—No difference in the dry weight and in the total nitrogen or phosphorus contents of the methylcholanthrene- or benzpyrene-induced tumors could be detected. Tests carried out on the homogenates showed that the tumors contained 15.75 ± 0.20 per cent solids; the solid material contained 14.19 ± 0.57 per cent nitrogen and 2.27 ± 0.01 per cent phosphorus. The amounts of nitrogen and phosphorus extracted by the various media are shown in Table 1. When controlled by the alkaline extraction media, the pH of the homogenates was between 7.7 and 7.8, and when not controlled it was between 6.9 and 7.0.

TABLE 2

NONPROTEIN NITROGEN INCREASE IN THE EXTRACT STORED AT DIFFERENT pH FOR 10 DAYS AT 0° C.

pH*	NPN mg/cc	ΔN NPN mg/cc	Protn. N mg/cc	Per cent loss protn. N
7.7† (initial)	0.92		0.458	
6.0	1.41	0.49	0.409	10.6
6.5	1.06	0.14	0.446	2.6
7.0	0.98	0.06	0.452	1.5
8.0	0.93	0.01	0.457	0.2
9.0	1.12	0.20	0.438	4.1
9.5	0.98	0.06	0.452	1.5
10.0	0.94	0.02	0.456	0.4

* Two of extract ml. + 5 ml. of Sørensen or carbonate buffer of 0.1 ionic strength, NPN determined, after the addition of 20 per cent trichloroacetic acid, on the centrifuged supernate.

† Values adjusted for 1/7 dilution of the fresh extract.

Extracts obtained with isotonic media gave low or negative values for desoxypentosenucleic acid. Upon dialysis against distilled water the extracts gave clear solutions, whereas dialysis against the veronal buffer of pH 8.6 gave a small gelatinous precipitate. This residue, rich in pentosenucleic acid, was soluble in ammonium hydroxide and 1 M sodium chloride.

Extracts prepared with the hypertonic medium gave higher desoxypentosenucleic acid values and a larger precipitate when dialyzed against the veronal buffer.

At pH 5.75 ± 0.05 all extracts showed an incipient cloudiness and at lower pH yielded a copious precipitate. Pentosenucleoprotein occurred in all the extracts and accounted for 10–15 per cent of the original amount present in the homogenate.

A study was made of the nonprotein nitrogen increase in the extract when stored at various pH for 10 days at 0° C. Extracts kept at a pH < 6.9 showed a gradual precipitation of protein. From the results recorded in Table 2 minima occur for the NPN content at two pH values. However, for storage of the extract, the lower pH 7.8–8.0 is to be preferred, since the more alkaline pH is likely to result in some protein denaturation.

Electrophoretic properties.—Typical patterns of the extracts obtained from the benzpyrene- and methylcholanthrene-induced tumors are shown in Figure 1. For both tumors the patterns are reproducible from preparation to preparation and are not altered by storage of the extracts in sealed ampoules at −25° C. for 2 months. The mobilities and relative compositions of the designated component groups are shown in Table 3.

Electrophoretic patterns of the first extracts were taken at a protein concentration of 1.9–2.6 per cent, while in the second extracts the concentration varied between 1.0 and 1.3 per cent. The protein concentration of the combined extracts was 2.0–2.1 per cent.

Patterns of the extracts prepared from the benzpyrene-induced tumors showed the presence of four clearly defined components—i.e., A', A, B, and C; while the patterns of the extracts from the methylcholanthrene-induced tumors showed three—i.e., A, B, and C. Also, different relative compositions for components B and C appeared in the patterns of the two tumor extracts. No differences in the electrophoretic patterns of the first and second extracts of the methylcholanthrene-induced tumors could be detected.

In the region of low mobility where the resolution is poor, the patterns were arbitrarily divided into two component groups, D and E. Consequently, the division of the area in this portion of the patterns was somewhat subjective, and the recorded values for the mobilities and relative compositions of these components have only qualitative significance.

The dissimilarity of the protein distribution in rat plasma to that in the tumor extracts is apparent when their respective electrophoretic patterns are compared (cf. Fig. 1). Several components with similar mobilities are present in both

patterns, but on the basis of this evidence alone they cannot be considered identical. Plasma from tumorous rats has approximately 8 per cent less γ-globulin than normal rat plasma and shows the presence of the "f-component" reported by Deutsch and Goodloe (5) in plasma of the Sprague-Dawley rats.

The rat plasma electrophoretic patterns were obtained on plasma (dil. 1:2) of both normal and tumor-bearing animals, collected as two separate pools made up of 2 ml. from each of twenty rats. The relative composition values recorded here for phoretic analysis. The results of a typical fractionation of a 125-ml. sample of a benzpyrene tumor extract containing 1.9 gm. of protein, using 0.5, 0.75, and saturated 3.87 M ammonium sulfate successively, are shown in Table 4. The electrophoretic pattern obtained for each fraction is shown in Figure 2, and the per cent recovery of each component within the fractions, as compared to the initial amount present, in Table 5.

The altered mobilities of the components within the fractions, as compared to those values obtained in the original extract, may be due to the

TABLE 3

MOBILITIES AND RELATIVE COMPOSITIONS OF THE ELECTROPHORETIC COMPONENTS IN THE TUMOR EXTRACTS WITH VERONAL BUFFER AT pH 8.6 AND IONIC STRENGTH 0.1

Extract medium	Extract	Mobilities† A'	A	B	C	D	E	Relative compositions A'	A	B	C	D	E
Methylcholanthrene-induced fibrosarcoma*													
Isotonic sucrose (4)	1st		8.1 ± 0.2	5.8 ± 0.1	4.1 ± 0.2	2.0 ± 0.2	0.9 ± 0.1		4.4 ± 1.5	15.4 ± 1.0	68.8 ± 0.8	6.9 ± 0.8	4.3 ± 1.1
Isotonic sucrose (4)	2d		8.6 ± 0.4	6.0 ± 0.1	4.3 ± 0.1	2.1 ± 0.1	0.8 ± 0.2		2.3 ± 1.0	16.3 ± 2.8	69.2 ± 0.9	7.7 ± 1.2	4.3 ± 1.1
Isotonic potassium chloride (4)	1st		8.0 ± 0.4	5.8 ± 0.1	4.0 ± 0.1	2.1 ± 0.1	1.0 ± 0.1		2.6 ± 1.7	14.4 ± 1.3	69.1 ± 1.5	8.9 ± 1.7	5.0 ± 0.1
0.5 M potassium chloride + 0.03 M sodium bicarbonate (4)	1st		7.6 ± 0.4	5.8 ± 0.1	3.9 ± 0.2	2.2 ± 0.1	0.8 ± 0.1		2.6 ± 1.4	8.6 ± 1.3	74.5 ± 2.5	8.8 ± 1.1	5.4 ± 0.8
0.5 M potassium chloride +0.03 M sodium (4) bicarbonate, pH 8.0	2d		7.2 ± 0.5	5.8 ± 0.1	3.8 ± 0.2	1.9 ± 0.1	0.9 ± 0.1		3.9 ± 1.7	9.4 ± 2.0	74.6 ± 1.8	6.8 ± 1.2	4.5 ± 1.0
Benzyprene-induced fibrosarcoma*													
Isotonic sucrose (2) or Isotonic sucrose (3) +0.03 M potassium bicarbonate, pH 8.0	1st + 2d	11.0 ± 0.3	8.5 ± 0.1	5.9 ± 0.1	4.3 ± 0.1	2.3 ± 0.1	1.1 ± 0.2	2.2 ± 0.5	8.2 ± 1.7	22.2 ± 1.9	55.2 ± 3.1	8.0 ± 0.5	3.9 ± 0.9

RAT PLASMAS

	Mobilities† f	Alb.	Globulin α_1	α_2	$\phi+\beta$	γ	Relative compositions f	Alb.	Globulin α_1	α_2	$\phi+\beta$	γ
Normal rat plasma	0	5.7	4.8	3.7	2.6	1.5	0	42.7	18.6	10.4	17.3	11.0
Tumorous rat plasma	7.0	5.7	4.9	3.9	2.3	1.4	6.9	42.0	18.0	11.3	18.7	2.8

* Results are expressed as the mean ± the standard deviation based on 4 or more values:

$$\text{St. dev.} = \sqrt{\sum \frac{d^2}{n-1}}.$$

† $cm^2/v/sec \times 10^{-5}$.

normal rat plasma correspond to those reported by Gjessing and Chanutin (6).

Ammonium sulfate fractionation.—The tumor extracts were fractionated at 0° C. with saturated ammonium sulfate solution adjusted to pH 7.0 with either ammonium or sodium hydroxide. The ammonium sulfate was added to the chilled extract by dialyzing it slowly through a rotating membrane in the manner described by McMeekin (16) and Theorell (26). The precipitates were collected by centrifugation at 4° C. for ½–2 hours, washed by suspension in the same concentration of ammonium sulfate used for precipitation, and collected as before. They were dissolved in 1 M sodium chloride, and aliquots were taken for electrophoretic analysis... fractionation procedure but could also result from the influence of the changed protein composition on the apparent mobilities.

Although relatively little material is precipitated at one-third saturation, the concentration of C in this fraction is high, i.e., 70–75 per cent. As the salt concentration was further increased, fractions containing correspondingly larger amounts of B were obtained. No apparent concentration or preferential separation of the other components was apparent in any of the fractions.

DISCUSSION

It has here been assumed that the extracted proteins are mainly cytoplasmic in origin and

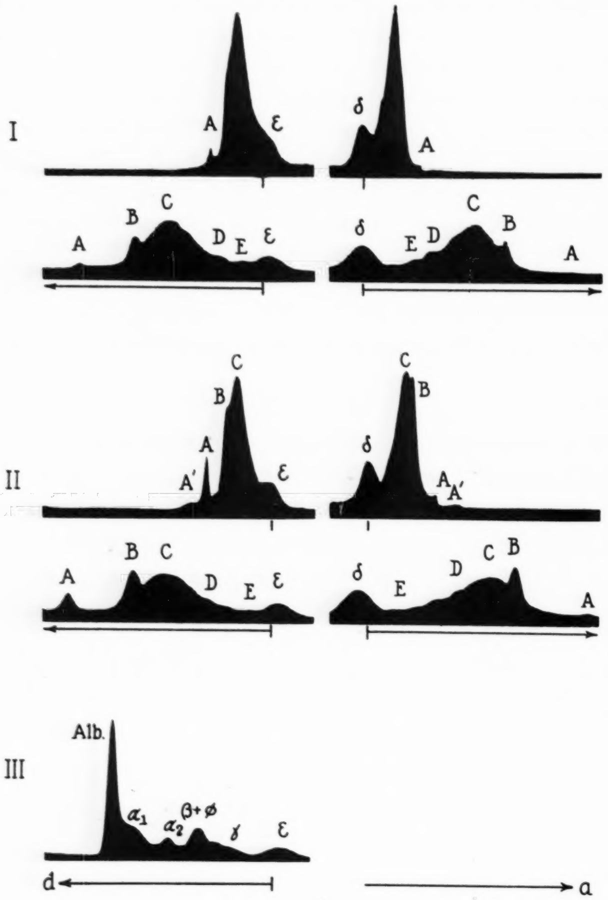

FIG. 1. ELECTROPHORETIC PATTERNS OF THE WHOLE TUMOR EXTRACTS AND OF NORMAL RAT PLASMA TAKEN IN VERONAL BUFFER, pH = 8.6, μ = 0.1 AT 4.7 VOLTS/CM

I. EXTRACT OBTAINED FROM THE METHYLCHOLANTHRENE INDUCED TUMOR - 60 AND 240 MINUTES

II. EXTRACT OBTAINED FROM THE BENZPYRENE INDUCED TUMOR 60 AND 240 MINUTES

III. NORMAL RAT PLASMA 300 MINUTES

that the amount of protein material leached from the nuclei, mitochondria, granules and microsomes or contributed by blood is small. Some lymph contamination of the extracts is possible. However, extracts of the tissue made with alkaline media brought considerable amounts of desoxypentosenucleoprotein into solution. Microscopic examination of smears prepared from these homogenates and residues indicated that some nuclear dissolution was taking place. Per gram of material extracted, these extracts also gave increased phosphorus and desoxypentosenucleic acid values and ultraviolet absorption values at 257 mμ. These effects were especially noticeable in the second extracts. The varying amounts of pentosenucleoprotein in all the extracts may indicate either an incomplete separation or a partial solution of the microsomes.

Because electrolytes tend to agglutinate the cell particulates (20), thereby making sharp centrifuge separation of the fractions difficult, sucrose was substituted in the extraction media (10). However, this substitution did not affect the gross protein yields obtained in the extracts. In order to obtain an extract with a protein concentration suitable for both fractionation and electrophoretic determinations, it was necessary to work with a concentrated homogenate. As efficient centrifugation of the particulates is dependent upon the viscosity of the solution, a 0.25 M sucrose concentration was used for extraction rather than the more desirable 0.88 M (10).

The most outstanding property of the tumor tissue extracts is the remarkable reproducibility of their electrophoretic patterns, both in the number

TABLE 4

MOBILITIES AND RELATIVE COMPOSITIONS OF THE ELECTROPHORETIC COMPONENTS OBTAINED IN THE FRACTIONS FROM THE BENZPYRENE TUMOR EXTRACT WITH VERONAL BUFFER AT pH 8.6 AND IONIC STRENGTH 0.1

Fraction	Conc. of ammonium sulfate used	Mobilities in $cm^2/v/sec \times 10^{-5}$						Relative composition					
		A¹	A	B	C	D	E	A¹	A	B	C	D	E
Initial extract	0	11.4	8.3	5.7	4.2	2.2	1.4	2.3	8.6	25.8	51.7	7.4	4.1
F-I	0.50 sat.	11.2	8.4	6.4	4.7	2.4	1.1	3.7	10.5	12.9	62.5	7.6	2.9
F-II	0.75 sat.	10.8	8.1	5.6	3.8	2.2	0.9	3.1	6.0	31.6	43.7	10.2	4.2
F-III	sat.	11.0	8.9	6.1	3.6	1.9	0.8	2.9	7.5	40.5	39.9	5.2	4.6

TABLE 5

PER CENT RECOVERY OF ORIGINAL EXTRACT ELECTROPHORETIC COMPONENTS WITHIN THE AMMONIUM SULFATE FRACTIONS

Fraction	Component						Per cent of total
	A¹	A	B	C	D	E	
Original extract	2.4	8.6	25.8	51.7	7.4	4.1	100
F-I	1.9	5.5	6.7	32.7	3.9	1.5	52.4
F-II	0.5	1.4	5.5	7.6	1.8	0.7	17.5
F-III	0.7	1.9	10.4	10.2	1.3	1.2	25.8
Total in fractions	3.2	8.8	22.6	50.6	7.0	3.4	95.7
Per cent loss or gain	+0.8	+0.2	−3.2	−1.1	−0.4	−0.7	−4.3

of components and in their relative compositions. However, the apparent complexity of the extracts and the relatively poor electrophoretic resolution obtained for some of the components leave much to be desired in the analysis of crude tissue extracts. The fast component of mobility -11.0×10^{-5} sq cm/v/sec obtained in the electrophoretic patterns of the benzpyrene-induced tumor extracts may correspond to hyaluronic acid (9, 13, 17). This material has been isolated from mucilaginous tumors and tumor fluids by Kabat (13) and by Meyer and Chaffee (17). These authors (17) claim that hyaluronic acid is elaborated by the tumor cells themselves. The complete solubility of the extract proteins in distilled water also suggests they are pseudo-globulins (8).

The electrophoretic studies made by Abrams and Cohen (1) of human lymphoid tissue and calf thymus, and by Roberts and White (21) of rat lymphoid and lymphosarcoma extracts, showed components with mobilities and relative compositions similar to those reported here. A more recent study (2) of a human lymphosarcoma tissue extract indicated the presence, in appreciable concentration, of a component having a mobility between that of D and E.

The presence of A^1 in the benzpyrene-induced tumor, and its absence in the case of the methylcholanthrene, is, therefore, of some interest. At the present time, no conclusions can be drawn from the differences reported here in the relative electrophoretic distributions of the components in the extracts obtained from the two induced rat fibrosarcomas. Whether the carcinogenic agents employed acted on the normal cells by two different pathways or whether two different cell types were involved in the malignant transformation cannot, at present, be determined. Histologically and morphologically, the cells of these two tumors appear identical. Although two similar neoplastic tissues have here been compared, investigations of this type are usually faced with the difficulty of obtaining suitable controls. This situation remains as the stumbling block in making any comparative studies with normal tissues. Certainly, further information concerning the tissue proteins of normal tissues is urgently needed before any generalizations are possible.

It might be of interest to compare the protein distributions of extracts prepared from histologically similar and equally malignant tumors which were induced by a wide variety of carcinogens and also to find out whether the same carcinogen can repeat the same type of transformation. The analysis of the protein distributions in histologically similar tumors of varying degrees of malignancy is also indicated. The number of pathways leading to a malignant transformation may be limited.

SUMMARY

Extracts prepared from two rat fibrosarcomas were examined by both physical and chemical procedures. The amount of nitrogen and phos-

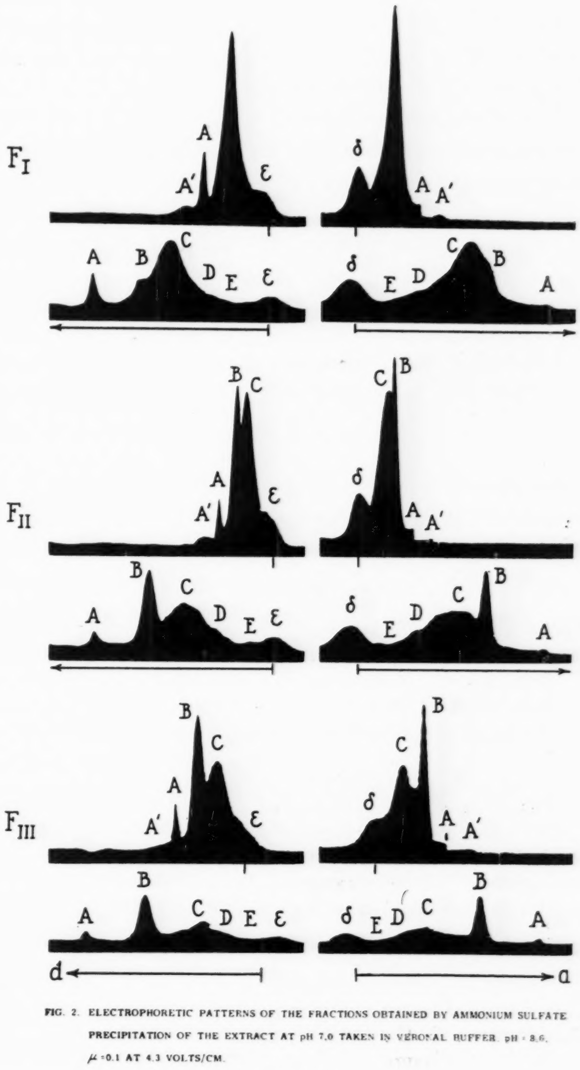

FIG. 2. ELECTROPHORETIC PATTERNS OF THE FRACTIONS OBTAINED BY AMMONIUM SULFATE PRECIPITATION OF THE EXTRACT AT pH 7.0 TAKEN IN VERONAL BUFFER pH = 8.6, $\mu = 0.1$ AT 4.3 VOLTS/CM.
F_I FRACTION FROM 0.50 SATURATION
F_{II} FRACTION FROM 0.75 SATURATION
F_{III} FRACTION FROM SATURATION.
ALL PATTERNS WERE TAKEN AT 60 AND 240 MINUTES

phorus extracted and the nature of the electrophoretic patterns of the extracts were independent of the extraction media employed. Three clearly defined electrophoretic components were observed in the methylcholanthrene- and four in the benzpyrene-induced tumor extracts. The number of electrophoretic components, their mobilities, and their relative compositions were readily reproducible from preparation to preparation, and no

changes in the electrophoretic patterns were detected when the extracts were stored at −25° C. for 2 months. If kept at 0° C., the extracts were most stable at pH 7.8–8.0.

Electrophoretic patterns taken on plasma of normal and tumor-bearing rats were similar, except that for the latter animals a slight γ-globulin decrease was indicated. The relative compositions of the electrophoretic components found in the tumor extracts and in normal rat plasma were dissimilar, but several of the components had similar electrophoretic mobilities in both patterns.

The apparent complexity of the extracts and the incomplete electrophoretic resolution obtained suggest that other components are associated with each peak. Fractions were prepared by ammonium sulfate precipitation, and these were electrophoretically examined

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The Urinary Excretion of Mercapturic Acids after Administration of Bromobenzene and 3,4-Benzpyrene*

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In a previous communication (1) it was shown that the rat forms some mercapturic acid from simultaneously administered radioactive L-cystine and bromobenzene. The major portion of the excreted mercapturic acid was derived from unlabeled tissue sulfur compounds. On the other hand, no evidence could be found for the excretion of a labeled conjugate from 3,4-benzpyrene and L-cystine. In addition, the distribution of labeled urinary sulfur was not affected by administration of the carcinogen.

These experiments have been repeated in the present study with labeled DL-methionine in place of L-cystine with essentially the same results. This approach did not eliminate the possibility that benzpyrene might have reacted with minute amounts of unlabeled tissue sulfur compounds. Consequently, the contribution of labeled tissue sulfur compounds to mercapturic acid formation has been investigated with particular reference to benzpyrene conjugation.

Sulfur compounds of the body of the rat were labeled with S^{35} by feeding radioactive DL-methionine over a period of 15 days. 3,4-Benzpyrene, anthracene, phenanthrene, or bromobenzene was administered. The urine was collected for a period of 48 hours and fractionated. A mercapturic acid arising from the conjugation of any of these aromatic compounds with labeled tissue sulfur was accessible to measurement by the methods of the radioactive tracer technic. The conjugates expected from bromobenzene and anthracene were detected in the chloroform fractions. Following the administration of phenanthrene, appreciable amounts of "chloroform-soluble" sulfur were also found. There was no evidence of the formation of a similar conjugate after the ingestion of 3,4-benzpyrene.

MATERIALS AND METHODS[1]

Male rats of the Yale strain, weighing from 200 to 400 gm., were employed. Collection of the urines was carried out as described previously (1). The basal diet for all experiments was as follows: casein, 6; dextrin, 36; sucrose, 19; lard, 19; cod-liver oil, 5; corn oil, 1; salt mixture, F.R.L. (5), 4; dry yeast, 8; Celluflour, 2.

The radioactive DL-methionine used in the first experiments was synthesized in this laboratory. In later experiments, methionine labeled with S^{35} was obtained from the Abbott Laboratories. The labeled methionine in both cases was shown by the constant solubility test (2) to contain no radioactive impurities. The specific activity of the compounds ranged from 0.93 to 2.7 counts/sec/μg of sulfur. Bromobenzene was redistilled prior to administration, and the fraction distilling at 42°–44.5° C. was used. Anthracene was obtained from Eastman Kodak and had a melting point of 216° C. Phenanthrene, m.p. 100° C., was purified through the picrate complex, followed by recrystallization from hot absolute ethanol. The 3,4-benzpyrene, m.p. 178°–179° C., was the same preparation employed in the previous investigation. The aromatic hydrocarbons, or halogen derivatives, were dissolved or suspended in warm corn oil and were administered by stomach tube in the amounts stated in the tables. In the experiments summarized in Tables 1 and 2 the basal diet was supplemented with 0.5 per cent cystine, and the DL-methionine was administered in a single intraperitoneal injection of a water solution. In the experiments of Table 3, 0.5 gm. of labeled DL-methionine was mixed with 100 gm. of the basal diet. The animals were allowed to eat the radioactive diet *ad libitum*. The specific radioactivity of the urinary total inorganic sulfate sul-

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[1] All melting points are corrected.

TABLE 1

The Effect of Ingested Hydrocarbons on the Urinary Partition of S^{35} following the Administration of Labeled DL-Methionine

Rat	DL-Methionine injected (mg.)	Hydrocarbon administered (mg.)	Days	Labeled total S (μg.)	Labeled total SO_4-S (μg.)	Labeled Neutral S: $CHCl_3$ extractable (μg.)	Labeled Neutral S: $CHCl_3$ non-extractable (μg.)
A	10.0*	None	2	425	181	0.6	223
B	10.0*	None	2	401	214	0.6	189‡
C	9.2†	Bromobenzene 150	1	490	270	5	199
D	9.2†	Bromobenzene 150	1	425	97	26	272
E	10.0*	Bromobenzene 150	2	720	253	28	373
F	15.0*	Anthracene 50	1			1.7	
G	10.0*	Benzpyrene 43	2	629	335	0.6	257
H	10.0*	Benzpyrene 57	2	311	163	0.8	148‡
I	10.2	Benzpyrene 50	2			0.3	
J	10.2	Benzpyrene 50	2			0.4	

* The specific radioactivity of DL-methionine was 0.93 counts/sec/μg S.
† The specific radioactivity of DL-methionine was 2.7 counts/sec/μg S.
‡ Value obtained by difference.

TABLE 2

The Excretion of Labeled Sulfur 24 Hours after a Single Injection of Labeled DL-Methionine in Per Cent of the Administered Dose

Rat	Hydrocarbon Administered	Labeled sulfur excreted in 24 hours: Total S (per cent)	Total SO_4-S (per cent)	Neutral S: $CHCl_3$ extractable (per cent)	Neutral S: $CHCl_3$ nonextractable (per cent)
A	None	9	4	<0.1	5
B	None	10	5	<0.1	5*
C	150 mg. bromobenzene	25	14	0.3	10
D	150 mg. bromobenzene	20	5	1.3	14
E	150 mg. bromobenzene	17	7	0.7	9
G	43 mg. 3,4-benzpyrene	15	8	<0.1	6
H	57 mg. 3,4-benzpyrene	7	4	<0.1	3*
I	50 mg. 3,4-benzpyrene			<0.1	
J	50 mg. 3,4-benzpyrene			<0.1	

* Value obtained by difference.

TABLE 3

The Effect of the Ingestion of Aromatic Hydrocarbons on the Urinary Sulfur Partition following the Dietary Intake of Labeled DL-Methionine*

Rat	Hydrocarbon administered	Days	Total S (mg.)	Total SO_4-S (mg.)	Neutral S: $CHCl_3$ extractable (mg.)	Neutral S: $CHCl_3$ nonextractable (mg.)
J	None	2	9.9	3.9	0.05	
	31 mg. bromobenzene	2	11.5	5.1	0.49	2.1
K	None	2	5.5	2.3	0.02	3.1
	35 mg. anthracene	2	11.3	4.4	0.43	6.3
L	None	2	9.9	6.7	0.01	1.2
	35 mg. phenanthrene	2	9.0	3.0	0.16	4.8
M	None	2	18.0	6.9	0.05	10.6
	50 mg. benzpyrene	2	10.2	3.5	0.08	4.8
N	None	2	10.5	5.7	0.04	3.7
	50 mg. benzpyrene +31 mg. bromobenzene	2	12.4	3.3	0.49	8.2

* The specific radioactivity of the diet was 0.015 counts/sec/μg S.

fur was determined daily. It usually attained a constant value with the diet within 5–6 days. After 13 days, food was withheld for 48 hours. Before the hydrocarbon was administered, the feeding of the radioactive diet was resumed for another 48 hours in order to minimize any adverse effects of the starvation period. Then the hydrocarbon was administered, the urine was collected, and the urinary sulfur partition was repeated.

The partition of the radioactive sulfur in the urine was determined essentially as previously described (1). Labeled total sulfur was determined on an aliquot by the Carius procedure. The remainder of the sample was boiled with acid and then extracted with chloroform. The labeled total inorganic sulfate was precipitated with benzidine from an aliquot of the chloroform-extracted, boiled urine. Another aliquot of the chloroform-extracted urine was oxidized by the Carius procedure or by alkaline fusion with sodium peroxide when the volume of the aliquot prevented the use of the former method. The difference between the radioactivity of this sample and that of the total inorganic sulfate was due to labeled neutral sulfur which was not extractable by chloroform. The remainder of the labeled neutral sulfur was determined by oxidation of the chloroform extract. This fraction contained the mercapturic acid sulfur which had been excreted in the urine.

Radioactivity measurements were made according to procedures already described (1). In the experiments of Table 1 the specific radioactivity of the injected methionine served as a standard for the estimation of the labeled sulfur in the urine. When the urinary partition of the sulfur derived from tissue compounds was studied (Table 3), the specific radioactivity of the inorganic sulfate fraction was determined and compared with the radioactivity of the other fractions indicated in the table. The values thus obtained were expressed in micrograms of sulfur. The specific radioactivities of the undiluted samples prepared from various urines ranged from 0.015 to 0.025 counts/sec/μg of sulfur. These activities were sufficient to allow the detection of 15–25 μg. of sulfur for a total counting rate of 50 per cent above the background radioactivity.

RESULTS AND DISCUSSION

The distribution of labeled sulfur in the urine following a single intraperitoneal administration of labeled DL-methionine is shown in Tables 1 and 2. These experiments were designed to reveal a possible effect of an aromatic compound on the metabolism of methionine before its conversion to cystine had been completed. The effect, if not extensive, might be masked by the formation of labeled cystine, which has already been shown to contribute to sulfur conjugates.

When no hydrocarbon was given, approximately 20 per cent of the injected radiomethionine sulfur was excreted in the urine within 48 hours. Approximately half of the labeled sulfur was accounted for as inorganic sulfate. The remainder was present as labeled chloroform-insoluble, neutral sulfur. The compounds in this fraction have not been identified.[2] The nature of the small but measurable amounts of labeled chloroform-extractable, neutral sulfur also remains to be determined. The radioactive sulfur of this fraction (less than 0.1 per cent of the total administered labeled sulfur) was shown by control experiments not to be due to traces of sulfate sulfur. The simultaneous administration of bromobenzene and labeled DL-methionine was followed by a marked increase of the labeled total sulfur in the urine. The output of radioactive sulfur was approximately twice that of rats which had received the labeled amino acid only. The rise of neutral sulfur was due chiefly to increased quantities of chloroform-insoluble sulfur (Table 2). This finding suggests the possibility that bromobenzene interferes with the metabolism of methionine.

The contribution of injected methionine sulfur to mercapturic acid formation within 24 hours averaged 0.7 per cent of the injected dose in three animals. The corresponding value for the contribution of cystine sulfur found in similar experiments was 4 per cent (1). The difference might be explained on the assumption that cystine is an intermediate in the formation of a mercapturic acid from methionine, the rate of conversion being the limiting factor.

The administration of 3,4-benzpyrene to two rats produced no marked effect on the urinary sulfur partition. In particular, there was no evidence for the presence of a conjugation product from DL-methionine and 3,4-benzpyrene in the urine of four rats.

Inasmuch as methionine can serve as the sole source of protein sulfur for the rat, it may be assumed that inclusion of the radioactive amino acid in the diet should label the labile sulfur compounds of the tissue within a few days. The attainment of constant specific radioactivity of the urinary sulfate may be considered to indicate that all tissue

[2] The chloroform-extracted urine was treated with unlabeled DL-methionine, benzoyl-chloride, and alkali at ice-bath temperature. N-Benzoyl-DL-methionine was isolated in the usual manner and was obtained pure, m.p. 149.5°–150.5° C., after two recrystallizations. A sample was oxidized to sulfate by the Carius method. It was shown to contain no radioactive sulfur.

compounds which make a major contribution to this fraction have been labeled.

In the experiments summarized in Table 3, equimolar amounts of the aromatic hydrocarbons or bromobenzene were administered to animals prepared as described above. The appearance of mercapturic acids after bromobenzene and anthracene is indicated by an increase in the chloroform-extractable neutral sulfur. A similar increase in the case of phenanthrene is suggestive of the *in vivo* synthesis of phenanthrylmercapturic acid. Although this compound has never been identified by isolation, its formation has been postulated chiefly on the basis of growth studies (3,4). The increase in the excretion of chloroform-extractable sulfur after administration of 3,4-benzpyrene was within the limits of error of the analytical procedure. When bromobenzene and 3,4-benzpyrene were administered simultaneously (Rat N), the carcinogen failed to decrease the extent of the formation of bromophenylmercapturic acid.

SUMMARY

Effects of bromobenzene, anthracene, and 3,4-benzpyrene on the metabolism of DL-methionine labeled with S^{35} have been studied in the rat. Measurements of the distribution of the radioactive sulfur in the urine indicated the excretion of a mercapturic acid from bromobenzene and anthracene but not from benzpyrene. The available evidence suggests that bromobenzene interferes with the metabolism of methionine.

The contribution of tissue sulfur compounds to mercapturic acid formation was estimated after the sulfur-containing compounds of the body of the rat had been labeled by feeding radioactive methionine over a period of 15 days. The presence of appreciable amounts of "chloroform-extractable" sulfur in the urine following the ingestion of bromobenzene and anthracene confirms the view that these mercapturic acids are largely derived from tissue sulfur compounds. The data suggest the synthesis of phenanthrylmercapturic acid *in vivo*. No evidence could be found for the conjugation of 3,4-benzpyrene with tissue sulfur. 3,4-Benzpyrene did not interfere with the *in vivo* synthesis of bromophenylmercapturic acid.

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Bilateral Ovarian Teratomas in a Mouse*

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Solid ovarian tumors of all types are very uncommon in the lower animals, and teratomas of the ovary are exceedingly rare. In 22,000 autopsies on mice, Slye, Holmes, and Wells (12) found only 46 with solid tumors of the ovary, and of these only one was a teratoma. Many of the mice they examined, moreover, were from stock having a high incidence of tumor formation.

The present paper reports the spontaneous occurrence of bilateral teratomas of the ovary in a mouse of the "Swiss" albino strain. The mass in the right ovary measured 23 × 19 × 20 mm. and that in the left ovary 17 × 10 × 10 mm. (Fig. 1). Both presented a mottled appearance suggesting, in the gross, the heterogeneous composition which was verified in histological sections. In the smaller tumor some remaining ovarian tissue was found, including one small follicle. The right ovary appeared to be almost entirely replaced by the teratomatous growth. The two tumors were similar in composition, consisting of several recognizable embryonic tissues in varying degrees of differentiation. There were large areas of nervous tissue (Fig. 3). Other areas contained glandlike structures, cysts filled with mucoid secretion, gastric mucosa, squamous cells forming epithelial pearls, and small nodules of cartilage (Fig. 5).

Extending over a considerable portion of the surface of one of the tumors was a deeply basophilic layer of cuboidal cells resembling the germinal epithelium which normally covers the ovary. In one locality, illustrated in Figure 4, the cells of this epithelium gradually became columnar and assumed the appearance of mucous cells. Extending inward from the mucus-secreting epithelium were tubular structures resembling typical gastric glands in which both chief and parietal cells were recognizable. The germinal epithelium of the ovary normally produces epithelial ingrowths which differentiate into totipotential primary oöcytes (4). If the layer of cuboidal cells covering this tumor was derived from the original covering of the ovary, then its smooth transition into gastric mucosa would seem to suggest that the cells of the germinal epithelium may be capable of differentiating directly into the various embryonic tissues found in ovarian teratomas.

Solid ovarian teratomas are generally thought to arise by parthenogenetic development of ovarian eggs (3, 5). Experimental evidence for this theory was derived from the work of Bosaeus (1), who removed unfertilized ova from frog ovaries, stimulated them with a needle to initiate development, and then reimplanted them into the same frog. In this way he produced teratomata. Similar experiments involving stimulating mammalian ova to develop parthenogenetically have not been done. However, fertilized mouse and rat ova have been successfully transplanted to several extra-uterine sites. The results of these experiments have differed considerably. Nicholas (10, 11) transplanted rat ova beneath the kidney capsule and to segments of gut. In these sites he obtained an accelerated growth of various embryonic tissues but no differentiation of placenta or fetal membranes. Some of these growths survived for as long as 54 days.

In experiments reported in detail elsewhere we have transplanted segmenting mouse ova to the anterior chamber of the eye, the abdominal cavity, under the capsule of the kidney, and into the substance of the brain (6, 7). In over 60 successful transplants to these several extra-uterine sites the resulting growth consisted of placental trophoblast and often a rudimentary yolk sac. These placentomas had a limited life-span corresponding in duration to the growth period of the normal mouse placenta. In none of these transplants did we observe a disorganized growth of tissues and organs such as occurs in teratomata.

Spontaneous parthenogenetic development of eggs has been observed several times in the ovaries of guinea pigs (2, 8). In those guinea pigs, de-

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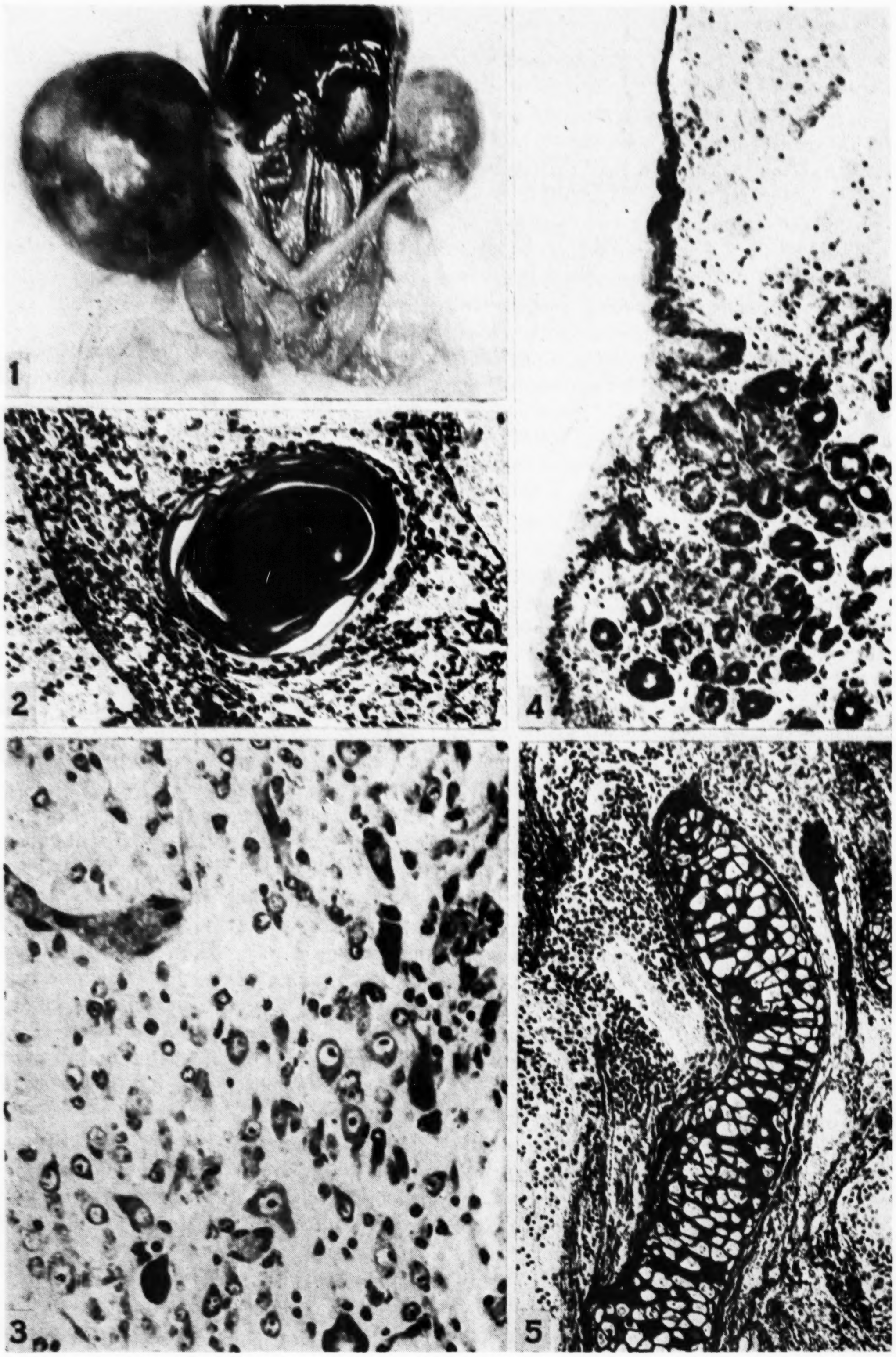

FIG. 1.—Photograph of the teratomas of the right and left ovaries *in situ*. ×1.5.

FIG. 2.—A concretion formed in an area of nervous tissue within one of the tumors. Toluidine blue. ×200.

FIG. 3.—Section of tumor showing an area of nervous tissue typical of that which was very abundant in the mass in the left ovary. Hematoxylin and eosin. ×300.

FIG. 4.—A section of unusual interest at the surface of the tumor. The cuboidal epithelium covering the tumor in the upper half of the figure gradually becomes a columnar epithelium of mucus-secreting cells in the lower half. Growing into the mass from this epithelium are tubular glands which at higher magnification showed all the characteristics of gastric mucosa. Eosin and methylene blue. ×120.

FIG. 5.—One of several bars of cartilage found in the tumors. Eosin and methylene blue. ×150.

velopment of the ova resulted in a growth of trophoblast and trophoblastic giant cells. To our knowledge no ovarian teratomas have been reported in this species.

Thus, eggs of experimental animals developing in the ovary and in other extra-uterine sites commonly produce placentomata without differentiation of the embryo, but under certain conditions they appear to be capable of forming teratomatous masses of embryonic tissues without development of placental structures. It is not known what determines which of these alternatives will be taken by an ovum.

Differentiation of both placenta and embryonic tissues and organs in the same mass has not been observed in these experiments. It is interesting that in the literature on human ovarian tumors there are numerous reports of choriomas and of teratomas, but there are scarcely any accounts of tumors which contained both fetal tissues and placental elements (9).

SUMMARY

A case of spontaneously occurring bilateral ovarian teratomas in a mouse has been described and discussed in relation to recent experimental work bearing upon the origin of teratoid tumors.

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The Effect of Storage at Low Temperature on the Viability of Several Avian Lymphoid Tumor Strains

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Several avian lymphoid tumor strains have been under study at this laboratory for the past few years. They originated from typical cases of naturally occurring visceral lymphomatosis. All are rapidly growing, highly malignant tumors, and a filtrable agent which reproduces the typical tumor has been demonstrated to be present in most of the tumor strains examined (4–8). However, the induction of tumors with filtrates appears to take a much longer incubation period than with cellular suspensions.

During the study of these tumors, it has been a routine procedure to freeze and to place in storage at low temperature samples of tumor from certain passages. At varying intervals after freezing, and for use in various experiments, the frozen tumor was removed from storage and implanted in chickens. The results of these inoculations have been summarized in this report.

MATERIALS AND METHODS

All tumor tissue used in these tests was propagated by the injection or implantation of a suspension of neoplastic cells. For tumor strains RPL 12, 14, 15, and 16 (6–8) such implantations were regularly made into the deep pectoral muscle. This resulted in an intramuscular lymphoid tumor which was palpable and which metastasized to the viscera. Tumor strains RPL 17, 18, 19, 20, and 21 (4–6) were propagated by intraperitoneal injection of a tumor suspension. The livers, in which diffuse tumors appeared, were used as a source of inoculum for passages by this route and for freezing and storage.

The tumorous tissue was sealed in Pyrex glass tubes with a gas-oxygen flame and frozen by the procedure described previously (3). All frozen tumor tissue was stored in a CO_2 icebox with a temperature range of from –65° C. at the top to –76° C. at the bottom of the box. Frozen tumor samples to be tested were thawed by placing the sealed tube in running tap water. The tumor was transferred aseptically to a mincer (13), processed, and suspended in 2 parts of 0.85 per cent saline. Preparations of tumors RPL 12, 14, 15, and 16 were injected into the deep pectoral muscle. All other preparations were administered intraperitoneally. The dosage varied from 0.1 to 1.0 ml. In general, it was adjusted to the size and age of the chicks used. The chickens used for experiments with strain RPL 12 varied in age from 30 to 182 days. Those used for all other experiments ranged from 7 to 30 days in age at the time of inoculation. With but few exceptions all chickens were from an inbred line of White Leghorns selected for susceptibility to lymphomatosis (15).

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RESULTS AND DISCUSSION

Results of the viability tests on nine tumor strains involving material from several passages in each strain are summarized in Table 1. A total of 1,350 chickens was used in 46 tests with fresh tumor material and in 89 tests with frozen tissue. No inoculation data were obtained on individual tumors prior to freezing; however, results obtained by the inoculation of chicks with fresh tumor of other birds from the same passage, or, in a few instances, from a prior passage, are given in the table for comparison with the data obtained with frozen and stored inocula. Since these tests extend over a period of about 6 years, the test chickens necessarily were obtained from many different matings; and, although all but a few were from line 15, it is a comparatively new line, and much variation was evident throughout the period of these experiments. This variation must be considered when comparing data of tests conducted at different times.

The length of the storage period was irregular, because the data include all results obtained with inoculation of frozen tumor, irrespective of the purpose of the particular inoculation.

The growth potential of the tumor samples was measured by the proportion of chickens in which the tumor grew (T/I: number of chickens with tumors / number of chickens inoculated) and by the incubation period. For strains RPL 12, 14, 15, and 16, in which inoculations were made into the pectoral muscle, the incubation period was arbi-

TABLE 1

The Activity of Avian Lymphoid Tumor Strains in the Fresh State and after Freezing and Storage for Various Periods of Time

Tumor strain	Passage number	Unfrozen inoculum T/I*	Unfrozen inoculum Incubation period (days)	Storage period (days)	Frozen and stored inoculum T/I*	Frozen and stored inoculum Incubation period (days)
RPL 12	4	2/2	11.0	384	8/8	5.6
				2,028	11/11	8.2
	12	5/6	7.4	102	8/8	9.8
				307	2/7	7.0
				1,951	11/11	8.0
	20	4/4	7.0	225	8/8	5.8
				1,869	11/11	8.0
	40	No test		203	7/7	9.4
	57	26/26	5.2	7	21/22	5.1
				81	7/7	8.0
				97	8/8	10.0
				956	7/7	8.0
				1,579	11/11	8.0
	87	7/7	7.0	50	7/9	
				77	7/7	9.0
				307	6/6	8.0
				1,273	11/11	8.0
	99	10/10	8.0	335	6/6	7.5
				635	7/7	11.0
				958	11/11	8.0
	101	No test		290	7/7	10.5
	126	5/5	7.4	401	7/7	10.0
	Average:	98.3 per cent	6.6		97.0 per cent	7.7
RPL 14	4	11/11	6.1	58	0/10	
	5	10/10	6.4	10	8/10	10.0
				1,433	10/10	14.8
	11	6/6	8.0	22	2/10	18.0
				177	2/12	10.0
	12	No test		1,176	10/10	11.0
	14	7/7	10.0	133	9/10	10.4
	Average:	100 per cent	7.3		60.9 per cent	11.8
RPL 15	4	13/16	6.8	60	0/10	
	4, a	15/15	7.0	33	4/11	16.0
	12	7/7	6.7	19	9/10	10.1
				174	6/12	10.8
				1,362	10/10	12.8
	13	No test		1,178	10/10	11.0
	16	7/7	7.0	231	10/10	10.0
				1,320	10/10	11.0
	Average:	93.3 per cent	6.9		72.1 per cent	11.3
RPL 16	3	10/10	6.2	28	9/10	14.8
				1,388	10/10	17.2
	10	7/7	5.0	19	6/10	14.3
				174	3/12	17.3
				1,321	12/12	17.2
	19	5/5	11.0	84	11/11	9.8
				329	6/6	
				1,231	11/11	12.1
	35	2/2	11.0	102	4/5	10.0
				130	13/15	10.6
				752	10/10	10.3
	Average:	100 per cent	7.3		76.7 per cent	13.8
RPL 17	3	17/17	24.1	360	13/15	26.9
				1,579	7/11	27.1
	4	8/10	17.8	343	14/15	36.0
				1,562	4/11	32.0
	9	15/15	19.0	125	6/11	19.1
				297	14/14	25.8
				1,516	1/11	37.0
	22	14/15	14.7	6	1/10	23.0
	28	14/14	13.1	1,356	0/10	
				1,418	3/7	18.0
	46	10/10	11.4	80	15/15	13.5
				80	13/15	13.9
				1,201	10/10	17.6
	Average:	97.0 per cent	16.0		69.7 per cent	23.0

* Number that developed tumors / number inoculated.

TABLE 1—*Continued*

Tumor strain	Passage number	Unfrozen inoculum T/I*	Unfrozen inoculum Incubation period (days)	Storage period (days)	Frozen and stored inoculum T/I*	Frozen and stored inoculum Incubation period (days)
RPL 18	6	10/10	12.5	215	6/10	58.8
				234	5/9	16.8
				532	1/6	17.0
	7	6/6	10.6	225	6/10	28.6
				873	6/10	11.3
	8	8/8	15.0	207	8/10	13.5
				312	6/13	24.3
	10	7/10	11.5	91	3/14	18.0
				116	10/10	12.4
				166	8/10	11.8
				221	8/12	12.1
	21	2/12†	8.0†	12	10/10	9.3
	23	3/10	10.0	28	9/13	11.6
	Average:	77.3 per cent	12.3		62.8 per cent	17.6
RPL 19	5	16/16	44.5	341	5/8	40.0
	6	16/19	43.2	250	8/9	40.0
	7	10/10	15.9	282	4/8	19.0
	9	10/10	17.0	250	8/8	16.0
	Average:	94.5 per cent	33.3		75.8 per cent	29.0
RPL 20	2	8/8	141.7‡	936	0/8	
	3	5/12	28.6	979	0/12	
	4	5/10	18.6	965	2/11	33.0
	5	6/10	17.1	911	2/8	11.0
	8	2/10	14.0	935	1/12	23.0
	Average:	52.0 per cent	20.9		16.1 per cent	22.2
RPL 21	1	11/18	25.3	939	1/10	28.0
	2	13/13	16.6	206	1/8	26.0
	3	9/10	16.8	993	1/12	25.0
	4	7/10	14.5	979	2/10	25.0
	5	9/10	16.1	167	0/8	
	8	4/9	11.8	924	3/12	19.0
	Average:	75.7 per cent	17.7		15.4 per cent	22.0

† Omitted from average; they were a different line of test birds.
‡ Omitted from average; atypical.

trarily defined as the number of days from inoculation to the development of a definitely palpable tumor; whereas for strains RPL 17, 18, 19, 20, and 21 it was defined as the number of days from inoculation to death, when tumors were grossly visible in the viscera at necropsy.

It is apparent from the data that all the tumor strains tested were able to survive freezing and storage under the conditions described; however, certain tumor samples apparently lost all their growth activity. One tumor sample from each of strains RPL 14, 15, and 21 and two from RPL 20 were inactive. Only one tumor growth was obtained with one frozen sample each from RPL 17 and 20 and with three samples of RPL 21. Thus, of the 50 frozen tumor samples tested, 10 had little or no activity and all but 3 of these were from strains RPL 20 and 21.

There was considerable variation among the several strains in the growth activity after freezing and storage. Almost all birds (97 per cent) inoculated with frozen RPL 12 material developed tumors which grew to a palpable size in a short time (7.7 days); whereas frozen RPL 20 and 21 inocula produced only a few tumor takes (average of 16.1 and 15.4 per cent, respectively). The average percentage of takes with frozen material for the remaining tumor strains ranged from 61 per cent (RPL 14) to 77 per cent (RPL 16). Likewise, the apparent loss in growth activity was highly variable among strains and also among samples of the same strain. All strains showed a decrease in the average percentage of takes when the tumor was frozen and stored. Strain RPL 12 showed the smallest (1.3 per cent) and strain 21 the greatest decrease (60.3 per cent). The average incubation period for the tumor strains was lengthened after freezing and storage, with the exception of one strain, RPL 19.

The reason for the low viability of strains RPL 20 and 21 is not readily apparent; however, since in the fresh state the same strains also had the lowest growth activity, and strain 12 the highest, there is a suggestion in the data that the ability of a tumor to survive freezing and storage is related to its original growth potential. It can only be

speculated, at present, whether this relation is due to better viability of the individual cells of strain 12 or to the possibility that fewer live cells are necessary to produce a tumor than in strain 20, since data bearing on this point are not now available.

To determine whether there was a change in the growth activity of tumors following long-continued storage, all tumors that had been tested at two or more storage periods were grouped according to the results obtained after the shortest period and those obtained after the longest period. An examination of these data (Table 2) reveals that there were no consistent nor significant differences between the average of the results of tumor tests after the shortest and longest storage periods. The tumor samples of RPL 12, 14, 15, and 16, which were stored the longest, actually produced a higher percentage of takes than did tumors of the same source stored a shorter time; but their capacity to induce tumors was reduced by a variable extent. There was some evidence to suggest that the ability to maintain the capacity for growth was related to the growth potential of the fresh tumor, as measured by the incidence of tumor takes and the incubation period. There was no evidence of a reduction in the capacity to induce tumors by long continuous storage at a low temperature.

Gye *et al.* (9) and Mann (10–12) found that mouse sarcomas and mammary carcinomas retained their power to induce tumors after storage at −79° C. This and other treatments of the tumor before inoculation led them to conclude that such frozen tumor tissue produced tumors, because of the presence of a virus and not because of the direct multiplication of tumor cells.

A viral agent is present in most, if not all, the lymphoid tumor strains reported herein (2, 5, 7), and no doubt the effect of the virus would have

TABLE 2

EFFECT OF LENGTH OF STORAGE PERIOD ON THE VIABILITY OF LYMPHOID TUMORS

		Shortest storage period				Longest storage period			
Strain numbers	Number of paired tests	Average days stored	Number of chicks	Average percentage tumors induced	Average incubation period	Average days stored	Number of chicks	Average percentage tumors induced	Average incubation period
RPL 12	6	188.3	59	98.3	6.6	1,609.3	66	100	11.0
RPL 14, 15, 16	8	64.4	76	77.6	11.4	1,123.0	85	88.2	13.6
RPL 17, 18	8	206.0	100	71.0	26.3	974.0	84	51.2	20.0
Totals:	22	149.7	235	80.0	15.5	1,201.7	235	78.3	13.1

however, the average of tests of all strains was slightly lower for the longer storage period. For strain groups RPL 12 and RPL 14, 15, and 16, the average incubation period was slightly greater for tumor tissue stored a longer time, but for groups RPL 17 and 18 this situation was reversed. This resulted in an over-all average incubation period of 15.5 days for tumor samples tested after the shortest storage period and 13.1 days for samples tested after the longest period. Thus, the interpretation of differences in tumor takes is contrary to those of incubation period and further serves to indicate that changes in the growth potential during storage at low temperature are indeed small and could not be detected by methods used in the studies here reported.

Results reported herein for strain RPL 12 are similar to those obtained earlier (3), which showed that freezing at a slow rate, as suggested by Breedis (1), and storing at low temperature had no significant detrimental effect on the capacity of the strain to produce typical tumors and subsequent death to the hosts. Other similar tumor strains, RPL 14, 15, 16, 17, 18, 19, 20, and 21, also survived freezing and storage at low temperature, been manifested if the surviving birds had been maintained for a much longer period. It has been shown (8) that about one-half of the birds which survived tumor implants would develop tumors of the viscera if they were maintained for about 1 year.

Reactions obtained following the injection of a cell suspension of an avian lymphoid tumor are distinctly different from those obtained after the inoculation of filtrates from the same tumor (7). The former produces tumors at the site of inoculation with or without metastasis to the viscera in a period of 1–3 weeks; filtrates produce only typical lymphomatous involvement of several visceral organs, and in some strains, osteopetrosis, irrespective of the route of inoculation—and then only after an incubation period of 8 or more weeks (2, 4, 5, 7). In this respect, the lymphoid tumor virus is quite different from the Rous sarcoma virus (14), since with the latter there is little difference in the incubation period of tumors induced by cellular suspensions and cell-free preparations; also, tumors appear at the site of inoculation irrespective of the type of inoculum. Data presented in Table 1 are based on tumors which developed in

a short time and are thus presumably due to a multiplication of the tumor cells injected.

More direct evidence that viable cells were responsible for the rapid growth of lymphoid tumors was obtained in an experiment in which tumor tissue was treated in various ways to break up all the cells. Six lots of sixteen chicks each were inoculated with strain RPL 12 tumor that had been (*a*) frozen rapidly and thawed once, (*b*) frozen rapidly and thawed twice, (*c*) frozen rapidly and thawed 3 times, (*d*) shell-frozen and dried under high vacuum from the frozen state, (*e*) processed in a Waring Blendor for 10 minutes, and (*f*) frozen slowly by the controlled procedure used in the storage of lymphoid tumors (1). All the sixteen chicks of the last lot which were injected with slowly frozen material developed tumors in a short time; however, none of the chicks of the other five lots, with the exception of one chick in lot 5, grew tumors at the site of inoculation. This experiment demonstrates that the lymphoid tumor cells will not remain viable after one cycle of rapid freezing and thawing, processing in a blendor, or lyophilization. Other experiments (unpublished) have shown that when a tumor cell suspension was irradiated with a dose of roentgen rays sufficient to kill the cells but not the virus, and injected into chicks, tumors developed in the viscera only after a long incubation period.

Thus, it may be concluded that when procedures are used which will disrupt or otherwise kill the tumor cells, such material will not produce tumors at the site of inoculation in a short time. Active virus is present in such preparations of some of the strains and is capable of inducing tumors only after a long incubation period. Therefore, the activity of samples of frozen and stored avian lymphoid tumors to induce tumors at the site of injection in a short time is due to the presence of viable tumor cells and not to the activity of a virus.

Experiments reported herein provide good evidence that lymphoid tumor cells survive slow freezing but are readily killed by rapid freezing and lyophilization. These results, together with those of Breedis (1), lend support to the hypothesis of Gye *et al.* (9) that the activity of rapidly frozen or lyophilized mouse tumor must be attributed to something other than viable cells.

SUMMARY

The growth potential of nine avian lymphoid tumor strains involving 50 samples was tested in the fresh state, and after freezing and storage at low temperatures, by inoculation of tumor suspension into a total of 1,350 White Leghorn chickens.

All tumor strains survived freezing and storage, but there was considerable variation among samples and strains in the viability of frozen tumors. There appeared to be a direct relation between the growth potential of tumors in the fresh state and that after freezing and storage.

There was no evidence of a change in the growth potential of frozen tumors during long continuous storage at temperatures of from −65° C. to −76° C. A tumor sample tested 2,028 days after freezing produced tumors in all chicks inoculated, in a period of 8.2 days (average).

Data were presented which show that the activity of avian lymphoid tumors which had been frozen slowly was due to the survival of viable tumor cells and not to the activity of a virus.

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The Growth Potentialities of Induced Skin Tumors in Mice

The Effects of Different Methods of Chemical Carcinogenesis*†

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In their original description of the induced skin tumors of the rabbit, Yamagiwa and Itchikawa (14) detailed several morphological types of neoplasm with varying life histories. These tumors have been analyzed even more closely by Rous and Kidd (12), who have shown that a series of graded lesions are produced by applications of either tar or pure hydrocarbons, ranging from warts that regress to malignant metastasizing tumors. In the case of the mouse, it has been observed many times that a typical sequence of induced hyperplasia gives way to papilloma formation and that malignant transformation of some of these papillomas occurs. Here, too, the phenomenon of regression has been recorded. It has been noted that different carcinogenic hydrocarbons give rise to a variation in the type of neoplastic skin lesions in the mouse; in particular, the potent 9,10-dimethyl-1,2-benzanthracene has been observed by Bradbury *et al.* (7) to give rise to more papillomas than the other compounds used; methylcholanthrene has been found to be an effective carcinogen when administered as a single large skin application (10), and the tumors so induced (6) have been found to be largely carcinomatous from the moment of their first macroscopic recognition. So far, no detailed survey of the tumors induced in the skin of the mouse, similar to that carried out for the rabbit, has been reported.

Since it was demonstrated by Mottram (11) that a single application of a carcinogenic hydrocarbon followed by repeated applications of croton oil was an effective method of tumor induction for the mouse's skin, this method has been used extensively in a study of the phases of carcinogenesis by Berenblum and Shubik (3–5). From these experiments, much support has been gained for the concept that carcinogenesis is a two-stage mechanism consisting of a specific and irreversible *initiating* phase, in which normal cells are converted to latent tumor cells that lie dormant until stimulated in the *promoting* phase to become morphological tumors; the promoting phase can be brought about by noncarcinogenic procedures such as applications of croton oil, but it remains an ill-defined process from the standpoint of specificity (13). In previous experiments (4), quantitative surveys of the relationship of the dosage of the carcinogen to the total number of tumors induced were undertaken. All the tumors induced in the course of these experiments were charted twice weekly and eventually recorded in terms of their total number. No distinction was made among the various morphological types, the majority of tumors described being, in fact, benign papillomas. The question of early-appearing lumps that disappeared within a matter of a few weeks was considered, and these were felt to be similar to non-neoplastic lesions described by Cramer and Stowell (8). It was also noted at that time that later regression of undoubted neoplasms did occur and that the rate of malignant transformation was low. Allsopp (1), in a preliminary report, has noted similar findings with this method of carcinogenesis. In the present investigation, a comparison has been made between the tumors induced by this croton oil method and those induced by repeated applications of pure carcinogenic hydrocarbons to see if, in fact, the former do have special characteristics.

MATERIALS AND METHODS

Some of these experiments were performed at the Sir William Dunn School of Pathology, Oxford, and the remainder in this department. Fe-

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male Swiss strain mice, obtained from the Medical Research Council, England, and male and female strain CF-1 mice, from Carworth Farms, were used. The mice were fed an adequate diet with water *ad libitum*. All solutions were applied to the interscapular region, which was kept clipped free of hair with scissors. Croton oil obtained from Boots Chemists, Nottingham, England, was used for all the experiments as a 5 per cent solution in mineral oil (liquid paraffin); the 9,10-dimethyl-1,2-benzanthracene was also dissolved in mineral oil; and both these solutions were applied with a fine glass rod. The methylcholanthrene was dissolved in acetone and applied with a glass dropper.

The individual tumors on each mouse were recorded on charts at intervals of 2 weeks, and final histological confirmation of their nature was obtained. In any attempt to record the life histories of induced tumors many difficulties arise, making it necessary to establish arbitrary criteria that are of value more from a comparative than from an absolute standpoint. The tumors induced by the croton oil method proved by far the easiest of the induced skin tumors to record, as there tended to be fewer of them, and malignancy, if it supervened at all, occurred late. If applications of pure hydrocarbons were continued, so many tumors were produced in close proximity to one another that individual observation became extremely difficult. Most important of all is the effect of the onset of malignancy, represented macroscopically by a greatly increased growth rate, infiltration of the surrounding tissues, often ulceration, and with it regressions of many of the other benign tumors. This regression of benign tumors subsequent to the onset of malignancy in an adjacent tumor may be due to the overgrowing and obliteration of the benign tumor, or it may be secondary to ulceration and necrosis of the skin, or, lastly, it might be some other type of unknown regression phenomenon. For the purpose of this investigation, no attempt was made to determine the cause for regressions occurring subsequent to the advent of malignancy, and only those growth phenomena occurring before this were dealt with. The macroscopic diagnosis of malignancy in the mouse's skin, based on the onset of rapid infiltrative growth and often typical ulceration with eversion and raising of the edges of the lesion, was subjected to continued histological confirmation, and the two methods were found to tally remarkably. Almost all the lesions reported as malignant were anaplastic squamous-cell carcinomas with the exception of three fibrosarcomas.

The tumors observed in this investigation were found to fall quite clearly into four categories based on their growth characteristics. The classification adopted was:

1. Regressions: This constituted the largest group, and only those tumors that were present at least 4 weeks and then disappeared were included. Certain tumors were more than once found to disappear and then reappear during treatment. These were not included under "regressions," but for the purposes of the present investigation they were placed under one or another of the appropriate headings. All the regressions mentioned here did not reappear during the course of the experiments.
2. Stationary papillomas: all those tumors reaching a fixed size and remaining so for the rest of the experiment. Most of these tumors were small sessile papillomas.
3. Vigorously growing papillomas: tumors that continued growing throughout the course of the experiment in a benign fashion. Most of these were of the pedunculated papilloma variety and reached a considerable size.
4. Malignant tumors: the criteria for which have already been mentioned.

EXPERIMENTAL

Group I.—Group I consisted of 300 female Swiss mice, approximately 12 weeks old, which received a single application of 9,10-dimethyl-1,2-benzanthracene followed, after an interval of 3 weeks, by applications of 5 per cent croton oil in mineral oil for 40 weeks. This group was subdivided into three groups of 100 mice each; Group Ia received 0.17 per cent, Group Ib, 0.5 per cent, and Group Ic, 1.5 per cent of the 9,10-dimethyl-1,2-benzanthracene in mineral oil.

In Table 1 the tumor incidence for these various groups is recorded in terms of the various types of tumor. As was previously observed (4), the total tumor incidence, using this method of tumor induction, bears a direct relationship to the dosage of carcinogen. It would seem, however, that this factor does not significantly affect the distribution of tumor types. This latter conclusion cannot be stated with absolute certainty, and larger groups of mice with the lower concentrations used are needed for conclusive proof. Of the total of seven malignant tumors recorded here, six were squamous-cell carcinomas and one was a fibrosarcoma.

Group II.—Group II consisted of twenty-eight male and female CF-1 mice receiving 0.15 per cent methylcholanthrene in acetone (Group IIa) twice weekly for 30 weeks, and of thirty CF-1 mice receiving a single application of 0.3 per cent methylcholanthrene in acetone followed by twice-weekly applications of 5 per cent croton oil in mineral oil for 40 weeks (Group IIb). The results for all the

mice in this experiment are recorded in Table 2. It can be seen that, of the twenty-three mice in Group IIa developing tumors, seventeen had malignant invasive growths, of which two were fibrosarcomas and the others squamous-cell carcinomas; the eight regressions recorded occurred in three mice bearing papillomas, whose skin ulcerated and formed a thick scab that at autopsy had a purely inflammatory basis; lastly, the twelve stationary papillomas occurred in three mice, two of which died in the early stages of the experiment. The average amount of time elapsing from the time of the first recording of the tumors to the assumption of obvious invasive growth was 7 weeks. After the onset of invasive growth many regressions occurred, but these were not recorded, owing to the many complicating issues already considered. No regressions of diagnosed malignancies were seen. Of the seventeen mice with malignant tumors five developed a small papilloma giving way almost immediately to an ulcerative lesion that ultimately developed all the characteristics of malignancy. The remaining malignant tumors developed in vigorously growing papillomas.

In Group IIb the tumor distribution was very similar to that obtained in the previous croton oil experiment. The two malignant tumors were squamous-cell carcinomas.

TABLE 1

DISTRIBUTION PATTERN OF TUMORS INDUCED BY SINGLE APPLICATION OF DMBA* FOLLOWED BY REPEATED APPLICATIONS OF CROTON OIL

Group†	Concentration* of carcinogen (Per cent)	No. of survivors at time of first tumor	Distribution of tumor types: Regressions	Stationary papillomas	Growing papillomas	Malignancies	Total no. of tumors	No. of tumor-bearing mice
Ia	0.17	99	12	23	13	1	49	29
Ib	0.5	93	59	27	12	0	98	47
Ic	1.5	100	91	57	23	6	179	66

* 9,10-dimethyl-1,2-benzanthracene in mineral oil applied once only.

† A single application of carcinogen followed, after an interval of 3 weeks, by applications of 5 per cent croton oil in mineral oil twice weekly for 40 weeks.

TABLE 2

A COMPARISON OF THE DISTRIBUTION OF TUMORS INDUCED BY MC* APPLIED REPEATEDLY, WITH THOSE INDUCED BY A SINGLE APPLICATION OF MC* FOLLOWED BY REPEATED APPLICATIONS OF CROTON OIL

Group	Treatment	No. of survivors at time of first tumor	Distribution of tumor types: Regressions	Stationary papillomas	Growing papillomas	Malignancies	Total no. of tumors	Number of tumor-bearing mice
IIa	0.15 per cent MC* in acetone twice weekly	28	8†	12	0	17	69‡	23
IIb	0.3 per cent MC* in acetone *once*, then 5 per cent croton oil in mineral oil twice weekly	30	12	15	5	2	34	19

* MC: Methylcholanthrene. † Due to ulceration.

‡ The extra number of tumors represented here and not accounted for in the tumor distribution disappeared subsequent to malignancy and are therefore not counted, as mentioned in the text. Results were at 40 weeks from the first application of MC in Group IIa and 40 weeks from commencement of croton oil in Group IIb.

Group III.—In Group III a total of ninety CF-1 female mice were used. All these mice received a single application of 1.5 per cent 9,10-dimethyl-1,2-benzanthracene in mineral oil; the mice were then divided into three equal groups and after an interval of 3 weeks treated as follows: Group IIIa received twice-weekly applications of 0.1 per cent 9,10-dimethyl-1,2-benzanthracene in mineral oil for 20 weeks; Group IIIb received twice-weekly applications of croton oil for 35 weeks, and Group IIIc received a mixture of 0.1 per cent 9,10-dimethyl-1,2-benzanthracene and 5 per cent croton oil in mineral oil for 20 weeks. This last group was instituted to determine the existence of any possible anti-carcinogenic action on the part of croton oil giving rise to the high regression rate.

The results for this group are recorded in Table 3, where it can be seen that the Group IIIb treated with a single application of carcinogen followed by croton oil has reproduced a picture very similar to that seen previously. The Group IIIa, receiving repeated applications of 9,10-dimethyl-1,2-benzanthracene, was rather different from Group IIa receiving only methylcholanthrene, in that no regressions were observed, more papillomas were induced, and all the malignancies occurring began in vigorously growing papillomas and not in ulcerated areas. Group IIIc, receiving both croton oil and

9,10-dimethyl-1,2-benzanthracene, showed an increase in the number of tumors over either Group IIIa or Group IIIb and, with this, a slightly increased regression rate. The cause of this becomes difficult to interpret and might presumably be due either to the elicitation by croton oil of added papillomas not possessing sufficient growth-potentiality to survive or to a specific anti-carcinogenic effect of this substance.

CONCLUSIONS

These experiments show without doubt that there is a considerable difference between carcinogenesis by a single application of a carcinogen followed repeatedly by croton oil and carcinogenesis by repeated applications of the carcinogen alone. These differences are threefold: there is, first, an apparently increased regression rate; second, a marked decrease in the number of malignant transformations; and, third, vigorously growing benign tumors, not noted following application of the carcinogen alone, and closely resembling the benign tumors of man, are induced. These differences can be summarized by saying that the croton oil method yields a graded series of lesions ranging from tumors that only persist for a matter of months and then disappear, on through those that only reach a limited size, to frankly invasive tumors, with intermediate types being represented. With the repeated applications of the carcinogen, various types of tumor are induced too; but eventually malignant transformation occurs in almost all animals and obscures the fate of some of the other lesions. In particular, the fate of the regressions becomes obscured. With the croton oil method these occur between the 12th and 25th week of the tumor's existence, and, thus, experiments using carcinogen continuously cannot be expected to demonstrate this. The malignancies reported from the use of the carcinogens alone occurred, on an average, after the tumor had been present 7 weeks; and, therefore, any further regressions after this time were obscured. Nevertheless, there is no doubt that regression of some of the benign lesions does occur after this time, although what proportion simply merge into the rapidly growing malignant lesions cannot be determined. Thus, no true comparison can be made. It would seem that the most reasonable explanation of this picture, within the limits of the previously proposed two-stage mechanism of carcinogenesis, is to consider *initiation* as by no means a uniform process. A single application of the carcinogen results in the conversion of a certain number of normal cells to latent tumor cells, and the nature and existence of these changed cells may be revealed by the application of a noncarcinogenic *promoting agent*, such as croton oil. This promotion treatment reveals a series of graded lesions, varying in growth-potential, as described. Continued application of substances possessing initiating activity eventually results in a uniform induction of malignancy. Initiation would therefore appear to be a process conferring increased growth-potential on cells, and this increased growth-potential may vary considerably.

Berenblum (2) suggested that carcinogenesis consists of three phases: the initiation and promotion stages, as already considered; and, in addition, a specific stage of "meta-carcinogenesis" concerned with the conversion of the benign papillo-

TABLE 3

A COMPARISON OF THE INDUCED TUMORS*

Tumors induced by: (*a*) DMBA* applied repeatedly, (*b*) a single application of DMBA* followed by repeated applications of croton oil, and (*c*) a mixture of DMBA* and croton oil applied repeatedly.

Group	Treatment	No. of survivors at time of first tumor	Regressions†	Distribution of tumor types: Stationary papillomas	Growing papillomas	Malignancies	Total no. of tumors	Number of tumor-bearing mice
IIIa	One application of 1.5 per cent DMBA,* then, after 3 weeks, 0.1 per cent DMBA* twice weekly	26	0	36	0	21	90	25
IIIb	One application of 1.5 per cent DMBA,* then 5 per cent croton oil twice weekly	30	10	23	7	0‡	40	16
IIIc	One application of 1.5 per cent DMBA,* then mixture of 0.1 per cent DMBA* and 5 per cent croton oil twice weekly	30	5	65	0	19	143	24

* DMBA: 9,10-dimethyl-1,2-benzanthracene. All solutions made up in mineral oil. Results at 38 weeks from beginning of experiment.

† Previous to the onset of invasive growth.

‡ Two malignancies appearing in 48th week from beginning of experiment.

mas to carcinomas. From the data presented in this investigation, the changes necessary to alter the distribution of the tumor types and thus influence the rate of malignant transformation seem to be identical with the change involved in initiation. The inclusion of additional stages, involving changes of a nature different from those already considered, does not seem to be indicated. In the case of the tumors induced with a single application of carcinogen followed by croton oil, their particular natures would appear to have been determined by the initial treatment. Increasing the dosage of single applications of the carcinogen to be followed by a promoting agent does not appear to alter the tumor distribution, whereas repeated applications of the carcinogen do. It might be presumed that the first application alters the susceptibility of the cells involved to the second.

In their most recent report, Friedewald and Rous (9) have studied in detail the fate and time of appearance of tumors induced in five rabbits treated twice weekly for 250 days with methylcholanthrene and then with intermittent wound healing (disking) for up to 5 years. They observed a linear accumulation of large numbers of tumors during the whole of this period, and, in attempting to explain this phenomenon, they found it necessary to modify the previous concept of the mechanism of carcinogenesis. They feel that the substitution of *latent neoplastic potentialities* for *latent tumor cells* is necessary to explain these new observations.

The present experiments are felt to remain consistent with the view that carcinogenesis is begun by the conversion of some few cells to latent tumor cells. This is demonstrated with clarity by the use of a single application of a carcinogen as the initiating agent, and undoubtedly the use of repeated applications would result in a conversion of most of, if not all, the cells in the area to latent tumor cells. It is therefore difficult to compare the two types of experiments, although in the view of the present author the linear accumulation of tumors over a period of time can be considered consistent with the previous concept of carcinogenesis, with the modification that the latent tumor cells do not constitute a uniform change and that they represent differing grades of growth-potential.

SUMMARY

1. Skin carcinogenesis by a single application of 9,10-dimethyl-1,2-benzanthracene or methylcholanthrene followed by repeated applications of croton oil has been compared with carcinogenesis by repeated applications of either of the hydrocarbons alone.

2. The croton oil method yields only a small number of malignant tumors and gives rise to a very high regression rate.

3. The tumors induced have been classified as either (*a*) regressions, (*b*) stationary papillomas, (*c*) vigorously growing papillomas, or (*d*) malignancies.

4. From a consideration of the results obtained it is suggested that the process of *initiation* should be considered as a graded one, inducing graded changes in growth-potentiality in the *latent tumor cells*.

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Response of Transplanted Skin of Newborn and Suckling Mice to Application of 20-Methylcholanthrene*

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Subcutaneous transplantation of skin results in the formation of epidermal cysts which ultimately undergo destruction (3, 4). Application of 20-methylcholanthrene to the epidermis of young adult mice previous to transplantation stimulated epithelial growth in cutaneous and subcutaneous grafts made from skin thus treated. These grafts were likewise destroyed, unless the epithelium had, in the course of painting, acquired neoplastic properties (8). Neoplastic growth was, however, readily obtained if skin of mouse embryos was exposed to carcinogens previous to transplantation (6, 10, 11). The change in transplantability following stimulation by a carcinogen seems of interest in view of the role attributed to the sebaceous glands in epidermal carcinogenesis (6, 9–12). We therefore decided to study the transplantability of neonatal skin at various stages of development and following treatment with a carcinogen.

MATERIALS AND METHODS

The experiments were carried out in mice of the Swiss strain, maintained on a stock diet of Purina Laboratory Chow and water *ad libitum*. Pieces of skin, about 2 mm. square, were transplanted into subcutaneous pockets at each side of the chest wall of the recipient. Newborn and 1-, 2-, 4-, or 7-day-old animals were used as donors and related animals 2–3 months of age as recipients. Untreated skin was used for the control series. In the test series, a 0.3 per cent solution of 20-methylcholanthrene dissolved in benzene was applied with a camel's hair brush No. 6 to the skin of the donors a few minutes before they were killed. The transplants, or remnants thereof, were removed after 2, 4, 7, or 10 days; 2, 3, or 6 weeks; and 3, 6, or 12 months. The tissues were fixed in Bouin's solution, imbedded in paraffin, cut serially, and stained with hematoxylin and eosin. Altogether, 76 control and 156 painted transplants were examined microscopically.

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Microscopic Observations

Control Series

Grafts of skin of newborn mice.—Two days after transplantation, many cells of the surface epithelium and of the developing hair follicles had perished, and keratin was sloughed off (Figs. 1, *a*, 2, *a*). The surviving epithelium underwent hypertrophy and mitotic proliferation. The epithelium of the hair follicles migrated toward the surface and furnished most of the new epithelial covering. Within 4 days the surface of the graft was re-epithelized, and epithelium grew out from the edges of the transplant. After 7 days epidermal cysts began to form, with walls composed of two or three layers of elongated squamous cells and debris and keratin in the center. The corium underwent atrophy or necrobiosis and was infiltrated by leukocytes, phagocytic cells, and fibroblasts. This reaction varied in degree and in the proportion of polymorphonuclear and mononuclear leukocytes. Within 2 or 3 weeks the cysts had closed, while in both the graft and the wall of the cysts hair appendages continued to grow. The cysts were filled with keratin and hair and were surrounded by a connective tissue capsule. Six weeks after transplantation, the epithelial lining of the cysts was atrophic, and the connective tissue capsule had become thicker and denser than before. Two types of reaction could be distinguished in the grafts: Either the pressure of the capsule led to shrinkage of the cyst and loss of lining epithelium and scarring, or a marked foreign-body reaction characterized by a fibroblastic tissue and many multinucleated giant cells developed (Fig. 3, *a*). This granulation tissue grew into the center of the cyst and destroyed its lining as well as the remnants of the transplant. Six weeks or 3 months after grafting, two of eight transplants had been replaced by a small granuloma containing debris, dead hair, and keratin, while the remaining six transplants still showed epithelium. All eight transplants permitted to stay in the recipient for 6 or 12 months were resorbed.

Grafts of skin of 1- or 2-day-old mice.—The

transplanted epidermis remained somewhat better preserved than that of the newborn mice. On the whole, however, the fate of the grafts and the reaction of the recipient were in kind and intensity comparable to the changes described in the previous group. Six weeks or 3 months after transplantation, complete or fragmented epidermal cysts were identified in eleven of sixteen grafts. After 6 months, remnants of cysts were observed in two of eight transplants. Twelve months after transplantation, none of the grafts was recovered.

Grafts of skin of 4- or 7-day-old mice.—These transplants were fairly resistant to the early injurious influences of grafting, although—in keeping with the advanced development of hair at the time of transplantation—much keratin and hair had been shed from the surface of the grafts. The epithelium of both the hair follicles and the surface showed better survival than that of the younger individuals. There was vigorous epithelial growth inside the grafts and at their margins. Two days after transplantation multicentric regenerative growth originating chiefly in the hair follicles caused early re-epithelization of the graft. After 4 days, marginal outgrowth was in progress, and after 7 days cyst formation was advanced. Here and there, at the junction of the graft and the newly formed epithelium of the cysts, small epithelial pegs extended into the surrounding connective tissue. Three weeks after transplantation most cysts began to disintegrate. No essential difference in the early reaction of the recipients to these transplants, as compared to the younger ones, could be established. However, after the cysts had formed, abundant granulation tissue developed, causing rapid breakdown of the cysts. Six weeks or 3 months after transplantation, only four of sixteen grafts containing epithelial elements were recovered. Six or 12 months after transplantation, the grafts had been resorbed or replaced by a fibrous button showing a center of keratin and debris.

TEST SERIES

Grafts of skin of newborn mice.—During the early stages following transplantation, painted epidermis showed better resistance to the injury of grafting than that of unpainted skin. Within 2 days the epithelium of the hair follicles and of the surface underwent hypertrophy and vigorous mitotic proliferation. The epidermis became thicker, and the appendages developed more rapidly than in the untreated grafts (Figs. 1, *b*, 2, *b*). Long epithelial tongues grew out from the margins of the grafts. Sloughed-off keratin was abundant. Four days after transplantation epithelial outgrowth was advanced, and after 7 days large epidermal cysts filled with extruded hair, keratin, and debris were found. The cyst wall was composed of five to six layers of hypertrophic epithelium. Many hair follicles had lost their hair and had replaced it with plugs of keratin. Within the next 2 weeks, the proliferating epithelium of the

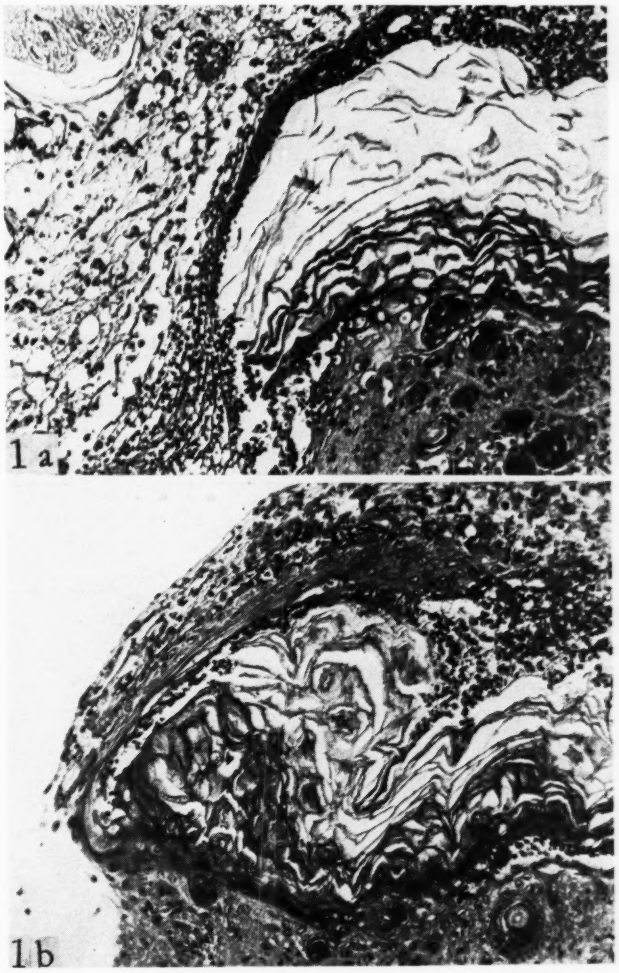

FIG. 1.—*a*. Untreated skin graft of a newborn mouse 2 days after transplantation. ×150. The surface layer is being sloughed off; there is slight reaction of the recipient. *b*. Painted skin graft of a newborn mouse 2 days after transplantation. ×150. Keratinization of the surface layer is advanced, and a large epithelial tongue grows out from the left margin of the graft.

hair follicles formed numerous pegs, composed of small hyperplastic epithelial cells with frequent mitoses (Fig. 3, *b*). The epidermal cysts continued to grow; sebaceous glands and abortive hair were present in the cyst wall. Infiltration of the graft by polymorphonuclear and mononuclear leukocytes was more conspicuous than in the nonpainted grafts. Gradually, connective tissue enveloped the the transplant and formed a capsule that increased

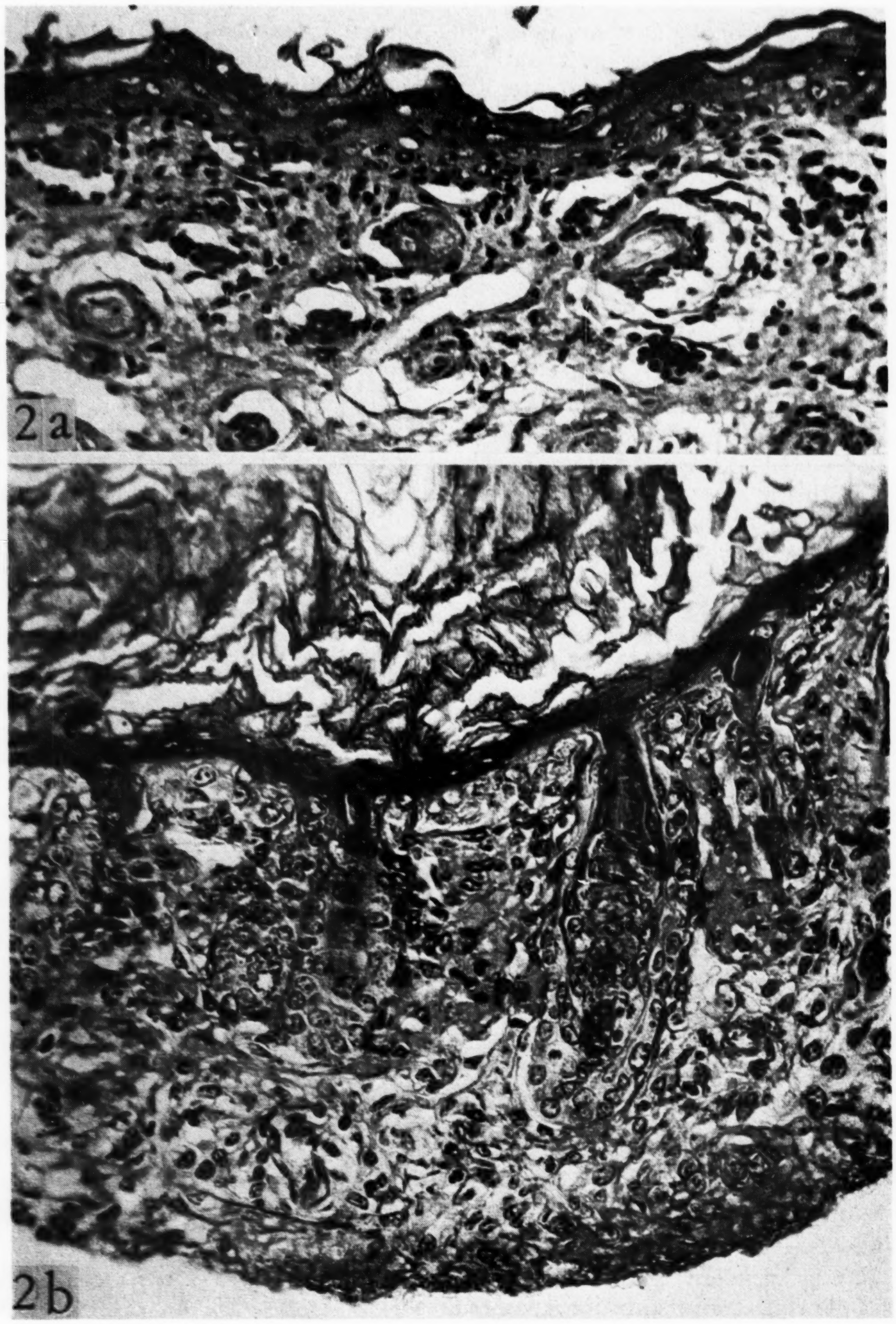

Fig. 2.—*a*. Same experiment as in Fig. 1, *a*. ×400. The graft is partly preserved, but there is little active growth. *b*. Same experiment as in Fig. 1, *b* × 400. Hyperplastic growth inside the graft is associated with good development of skin appendages; there is marked mitotic proliferation of the epithelium.

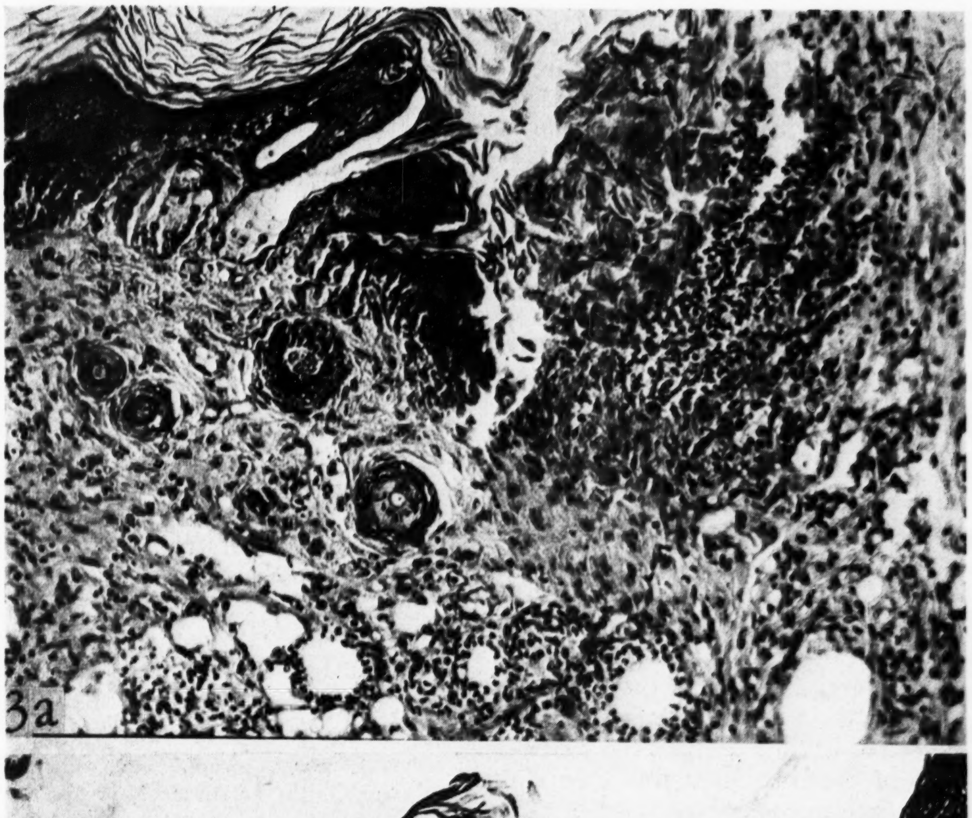

FIG. 3.—*a*. Untreated skin graft of a 1-week-old mouse 3 weeks after transplantation. ×200. A cyst originating from the margin of the graft is in an early stage of disintegration; there is a foreign body reaction with multinucleated giant cells. *b*. Painted skin graft of a newborn mouse 10 days after transplantation. ×200. The illustration shows the margin of a cyst at its insertion at the graft. Keratinized hair follicles are seen at the edge of the graft, and hyperplastic epithelial pegs are present in the cyst wall.

in thickness and density as time went on. Three weeks after grafting some cysts had been eroded, and a pronounced foreign-body reaction was noted in the vicinity. Other transplants and the attached cysts underwent shrinkage. Six weeks or 3 months after transplantation, epidermal cysts or remnants of such cysts were observed in eleven of sixteen transplants. Six or 12 months after grafting, no epithelial elements were found.

Grafts of skin of 1- or 2-day-old mice.—As a rule, growth and regressive changes in the transplant resembled those described in the preceding paragraph (Fig. 4, *b*). Six weeks or 3 months after transplantation, epithelial fragments were noted in 15 of 32 grafts, while 17 had been replaced by granulomatous tissue. Six or 12 months after grafting, scar tissue containing debris and keratin indicated the former site of the transplant.

Grafts of skin of 4- or 7-day-old mice.—During the early stages following transplantation many cells of the basal layer and of the hair follicles remained intact. The surviving epithelium gave rise to regenerative growth, which was more vigorous than in the corresponding nonpainted grafts. Re-epithelization of the transplant was thus accelerated, but less conspicuously so than in the painted grafts of skin of the younger mice. The outgrowing epithelial tongues were shorter but thicker than those seen in grafts of painted skin 1 or 2 days old at transplantation. Within 2 weeks large epidermal cysts were found filled with much keratin and hair. The cyst walls consisted of several layers of keratinizing epithelium. There were few appendages; if present at all, they were seen at the border between the original transplant and the outgrowing epithelium. Likewise in this area, but also in the transplant itself, numerous, large, proliferating epithelial pegs with keratotic centers were observed. Leukocytic and lymphocytic infiltration of the grafts was heavy. Three weeks after transplantation these changes were far advanced (Fig. 4, *a*), and a thick connective tissue capsule surrounded the epidermal cysts. Six weeks after grafting most cysts were broken up, and an abundant connective tissue growth with multinucleated giant cells was noticeable in the vicinity of bulky accumulations of keratin and debris (Fig. 4, *c*). Six weeks or 3 months after transplantation 9 of 40 grafts still showed remnants of cysts; others consisted of small granulomas surrounding inspissated debris, hair, and keratin, while still others had been absorbed. After 6 or 12 months, no epithelial elements could be detected. One mouse killed at the age of 12 months had developed a tumor at the site of transplantation. This growth proved to be a spindle-cell sarcoma, not arising in the transplant itself, but in the subcutaneous tissue of the recipient.

DISCUSSION

In syngenesiotransplants[1] of skin of newborn and suckling mice, epithelial growth increased with increasing age of the donor and as the hair follicles developed. The older the tissue at the time of grafting, the less pronounced was the new formation of appendages, the larger was the amount of hair and keratin within the cysts, and the more vigorous was the reaction of the recipient. Consequently, cysts derived from 7-day-old skin disintegrated earlier than those derived from skin 4 days old and younger at the time of transplantation.

The less active growth of grafted skin of newborn and of 1- or 2-day-old mice, as compared with that of 4- or 7-day-old mice seems contrary to expectation, since young tissues are considered to be endowed with greater growth capacity than older ones (4, 5). However, regeneration as well as outgrowth of the epithelium from the graft are not only contingent upon the inherent growth capacity of the regenerating tissue but also on the number of cells available. In mice below the age of 1 week, hair follicles are small and not yet fully developed. As the skin approaches full development, more cells are on hand which will respond to the stimulus of transplantation than in the epidermis of the newborn. Moreover, in the latter, the inherent tendencies to grow and to develop may possibly outbalance the requirements of regenerative growth created by experimental interference with the physiologic processes. As observed also in autogenous grafts, these developmental potencies seem to be pronounced (10, 11). The epithelium growing out from a graft of newborn skin had the ability to form hair follicles, sebaceous glands, and hair, although these potencies are lost with increasing age of the donor. Since hair will not develop without participation of dermal papillae, some equivalents of the latter presumably arise from small amounts of corium growing out with the epithelium.

20-Methylcholanthrene applied to the skin of mice immediately preceding transplantation stimulated growth of the epithelium and promoted the development of hair follicles and sebaceous glands. Epithelial migration was most marked in the epidermis of the newborn, and the epidermis

[1] These grafts belong to the group of homologous transplants, but the relationship between host and donor is closer than in the usual type of homoiotransplantation, host and donor being closely related individuals—e.g., brother and sister.

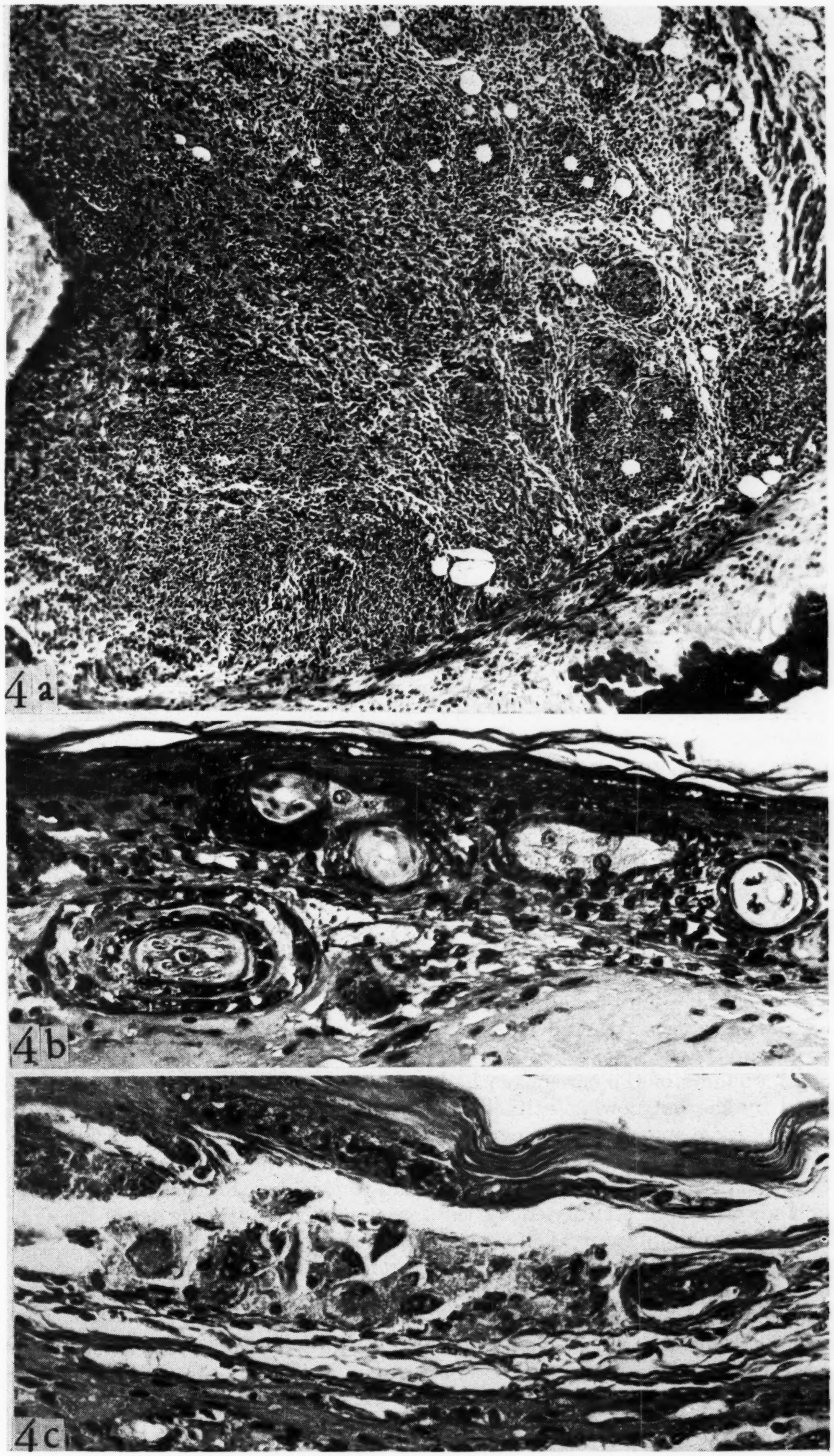

FIG. 4.—*a.* Painted skin graft of a 7-day-old mouse 3 weeks after transplantation. ×100. At the margin of the cyst deep epithelial pegs show marked leukocytic infiltration. *b.* Painted skin graft of a 1-day-old mouse 10 days after transplantation. ×400. Part of the cyst wall showing new formation of sebaceous glands and abortive hair. *c.* Painted skin graft of a 7-day-old mouse 6 weeks after transplantation. ×400. The cyst wall shows advanced foreign body reaction near deposits of keratin.

of 1-, 2-, or 4-day-old mice reacted with more vigorous growth than that of 7-day-old mice. The maximum of growth stimulation was represented by hyperplastic epithelial pegs arising from hair follicles and extending into the corium of the transplant; however, no carcinomas were found. The older the skin at the time of transplantation, the more intensive was the response of the recipient leading to destruction of the graft.

According to the present results, the epidermis of the newborn mouse is susceptible to stimulation by 20-methylcholanthrene. The fact that carcinomas did not develop cannot serve as proof to the contrary, since grafts of older epidermis did not show cancerous growth either. One reason for this failure may be the comparative weakness of the stimulus applied. In addition, factors inherent in the nature of the present experiment and related to the poor transplantability of skin in general opposed continuous growth of the grafts, both painted and unpainted. In this connection, the behavior of tarred skin transplanted into the anterior chamber of the eye is of interest (1). This environment is more favorable to successful grafting than the subcutaneous tissue, because here the reactive processes of the recipient are at a minimum (2). Nevertheless, growth of these transplants was only moderately stimulated by the application of tar, and in the end the grafts were destroyed (1).

Our observations of increased growth in the painted transplants associated with intensification of development of hair follicles and sebaceous glands may be compared to those made in grafts of embryonal skin treated previous to transplantation with a 1 per cent solution of methylcholanthrene. Under these conditions, the development of skin appendages was of the same order as in the controls, if small amounts of the carcinogen had become encysted by the outgrowing epithelium, while encystment of large amounts of the carcinogen caused destruction of the sebaceous glands (6, 10, 11). In our experiments, the development of sebaceous glands was even stimulated by the carcinogen. As also seen in guinea pigs (7), a weak solution of the carcinogen may stimulate the development of appendages, while strong solutions exert a harmful effect. In addition, the response of a tissue to a stimulus depends upon its physiologic age (5). Those processes which are in progress or impending at the time when the stimulus is applied are promoted most conspicuously. Correspondingly, the marked growth tendency of embryonal epidermis becomes accentuated if a graft of such skin is exposed to a carcinogenic agent. This growth stimulation may, under favorable conditions, lead to neoplastic growth (6, 10, 11). On the other hand, in newborn skin developmental processes are in progress. In grafts taken during this period, methylcholanthrene will promote not only processes of growth but also those of differentiation. After the appendages have reached their full development, methylcholanthrene will exert its growth-promoting effect on the epithelium rather than enhance development. Besides epithelial cell growth and development, migration of cells from the transplant plays a role in determining the fate of the latter. The epithelial hyperplasia produced by the carcinogen may partly account for the rapid outgrowth of epithelium and the accelerated formation of cysts in transplants of skin up to 3 days of age. At this age there seems to be an optimum in the rate of outgrowth. In painted grafts 7 days old at transplantation, many epithelial cells were present; yet, cyst formation was slow as compared with conditions in grafts of younger skin. However, widespread keratinization, occurring in the transplanted epidermis under the influence of the carcinogen, may have inhibited the movement of the epithelium. Previously, we reported a similar inhibition of epithelial migration over the base of wounds made in skin painted with a carcinogen (7). This inhibition might have been due to changes in the wound-base, to specific injurious effects of the carcinogen on the epithelial cells, or to increased keratinization of the epithelium. In the light of the present observations, the last-mentioned mechanism seems largely responsible for the decreased movement of epithelium painted with carcinogenic agents.

SUMMARY

In subcutaneous syngenesiografts[1] of skin of newborn or suckling mice, epithelial growth and migration were intensified, and cyst formation was accelerated, as the age of the donor increased. Conversely, the ability of the outgrowing epithelium to form hair follicles and sebaceous glands decreased with advancing age of the skin at the time of grafting. The reaction of the recipient was more pronounced against transplants of older skin than against those of younger skin, but ultimately all grafts were destroyed.

A solution of 0.3 per cent of 20-methylcholanthrene applied to the skin of newborn mice previous to grafting stimulated epithelial proliferation and migration and the development of appendages. These effects decreased with the increasing age of the donor. In grafts of 7-day-old skin, pronounced keratinization interfered with

the migration of the epithelium and the ensuing cyst formation. The reaction of the recipient was more vigorous against painted than against non-painted tissue. No carcinomas developed in the painted transplants, and the grafts were finally destroyed.

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The Effect of Low Temperature on the Morphology and Transplantability of Sarcoma 37*

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The first demonstrations that frozen tumor material can be successfully transplanted were reported by Michaelis in 1905 (31) and by Ehrlich in 1907 (14). Subsequent investigations (4, 5, 6, 7, 11, 15, 18, 23, 24, 25, 28, 32, 35, 36) have shown that tumor material stored at low temperatures for periods up to 2 years can be successfully transferred to animals. The temperatures used in these investigations ranged from $-8°$ C. to $-253°$ C.

Only a few studies (4, 5, 6, 7, 23, 32, 36) have taken into account the importance of the rate of freezing, and in these it has been reported that a slow lowering of the temperature results in a higher percentage of tumor "takes" than rapid freezing. Investigations into the physiological effects of low temperature (9, 10, 13, 19, 22, 27, 33, 34, 37, 39) have indicated that death at lower temperatures in normal tissues, is, in most instances, due to mechanical injury incident to either the formation or the melting of ice crystals; and animal or plant cells survive more readily when freezing is so rapid that an amorphous state of the water results.

Gye (17) has recently pointed out that various investigators have considered the possibility that the successful transfer of frozen-thawed tumor material might constitute proof of the transfer of a virus as a "continuing cause" of cancer. Yet many of these reports contain no description of the microscopic characteristics of the frozen material used for animal inoculation. Recent investigations by Gye *et al.* (17, 18) have shown that not only frozen material but also frozen-dried tumor can be successfully transferred. These workers conclude that cells cannot survive such treatment and that their observations are proof that tumor is being produced *de novo* in the host by a virus present in the inoculum.

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We have recently demonstrated that normal thyroid tissue can be successfully autotransplanted after freezing to $-70°$ C. or $-190°$ C. (3, 4). In these transplants not only cells but anatomic units, the thyroid follicles, survived. In light of these observations, it was decided to further investigate the effects of low temperature on tumor cells.

In the present experiments we have studied the effect of freezing and thawing on the percentage of tumor "takes." At the same time, the microscopic characteristics of the previously frozen inoculum and of the resulting tumor were compared with those of the original tumor (Sarcoma 37). In other experiments, a series of short-period studies, comparing transplants of unfrozen and frozen-thawed tumor, was carried out to further test the observation reported by Gye *et al.* (17) that visible tumor cells could not be found in transplants of frozen-thawed material a short time after inoculation.

Furthermore, if the transfer of frozen tumor is successful only because of the passage of a virus, it would not be expected that several repeated freezings and thawings would completely destroy the virulence of the material. Some previous reports indicate a loss of potency with such treatment, and data are presented here bearing on this point. Finally, in the course of these experiments additional data were obtained which indicate an effect of freezing on subsequent generations of tumor transplants.

MATERIALS AND METHODS

These experiments were carried out in mice of the Rockland, CaF-1, and CFW strains, as well as in a strain of hybrid albino mice constituting a stock colony in this laboratory and designated St.L.U. All mice were fed Purina Laboratory Chow and allowed water *ad libitum.*

The tumor used in these investigations was originally obtained from the National Cancer Institute and designated as Sarcoma 37. The inoculum consisted of a mince, prepared by cutting the

tumor into small fragments and passing these through a syringe several times. The needle size was gradually reduced until the mince would pass readily through a 24-gauge needle. No fluid was added, and each animal received 0.1 cc. of the inoculum subcutaneously.

The temperatures to which the tumor was subjected were 0° C., −30° C., −70° C., and −190° C. Exposure to 0° C. was obtained by placing tumor mince in an electric refrigerator; a deep freeze unit was used for exposure to −30° C.; an insulated box containing dry ice provided a temperature of −70° C.; and liquid nitrogen was used to obtain a temperature of −190° C. Tumor mince was placed in sterile 20-cc. vials and stored at these various temperatures for periods ranging from 1 day to 5 months. Thawing was accomplished by allowing the refrigerated tumor tissue to come gradually to room temperature.

The effect of refrigeration on the growth of transplanted Sarcoma 37.—The data presented in Table 1 show the effect of various refrigeration temperatures and periods on the latent period and the percentage of takes. The control data represent 58 transplant generations carried out in the four strains noted previously. The average latent period varied between 10 and 15 days and the percentage of "takes" between 83 and 100 per cent. These results show that strain specificity was not a critical characteristic of this tumor, although it appears to grow somewhat less readily in St.L.U. mice than in the other strains. When the tumor was stored at 0° C., there was no effect on the latent period of the tumor. The percentage of "takes," however, is inversely proportional to the length of time of storage. This observation cannot be attributed to a strain difference, as the inoculation of CaF-1 mice with material stored for only 24

TABLE 1

THE EFFECT OF FREEZING TEMPERATURE AND DURATION OF REFRIGERATION ON THE GROWTH OF TRANSPLANTED SARCOMA 37

Strain	Number of mice	Duration of refrigeration	Average latent period*	Percentage "takes"
		Group A (controls)		
St. L.U.	112		13 days	83
Rockland	167		15 "	96
CaF-1	163		13 "	96
CFW	40		10 "	100
		Group B (experimental: 0° C.)		
CaF-1	15	1 day	14 days	67
CFW	10	3 days	10 "	20
CaF-1	12	7 "	13 "	41
CFW	10	10 "		0
CaF-1	14	13 "		0
		Group C (experimental: −30° C.)		
Rockland	37	1 day	20 days	94.6
CaF-1	16	7 days	18 "	81.3
CaF-1	12	14 "	14 "	100
CaF-1	15	27 "	8 "	100†
CaF-1	24	41–62 "	9 "	100†
		Group D (experimental: −70° C.)		
CaF-1	19	1 day	12 days	100(11)‡
Rockland	34	1 "	14 "	76.5
St. L.U.	9	1 "	14 "	100
CaF-1	20	14 days	16 "	75
CaF-1	16	27 "	12 "	100
		Group E (experimental: −190° C.)**		
CaF-1	19	1 day	12 days	100(21)‡
CaF-1	16	27 days	20 "	100†

* Time elapsed between inoculation of tumor material and the appearance of a palpable nodule.

† These tumors grew for a time and then all gradually regressed. At the end of approximately 25 days only a small fibrous nodule remained at the site of injection.

‡ Per cent regression after an initial growth period.

** Tumor tissue placed at −190° C. for 10 minutes and then stored at −70° C.

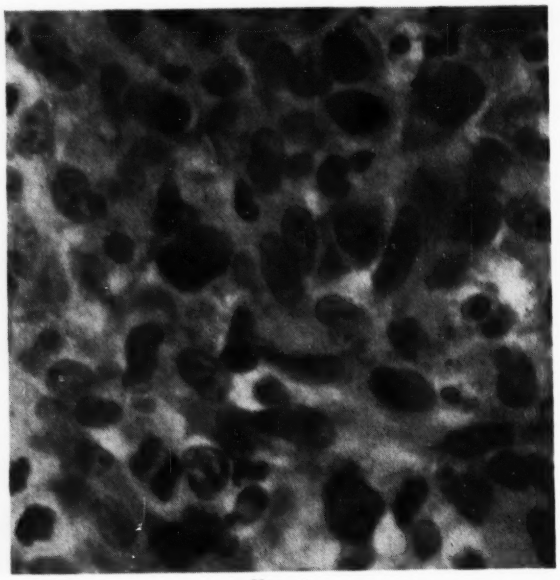

Fig. 1

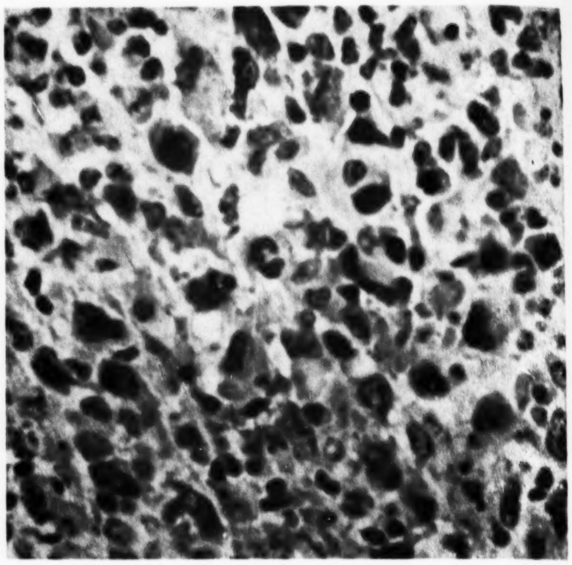

Fig. 2

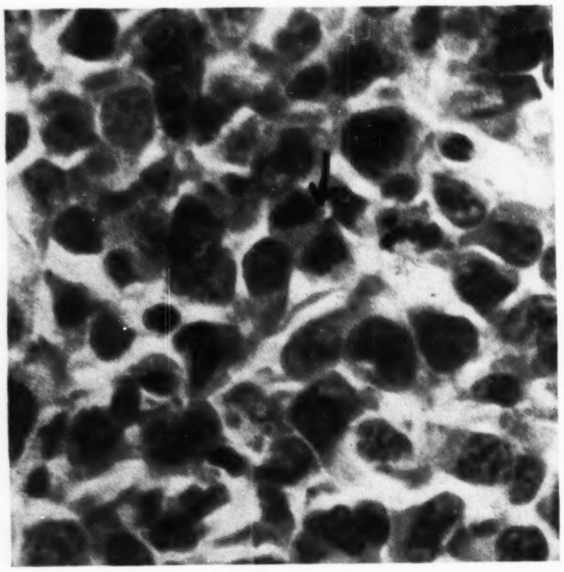

Fig. 3

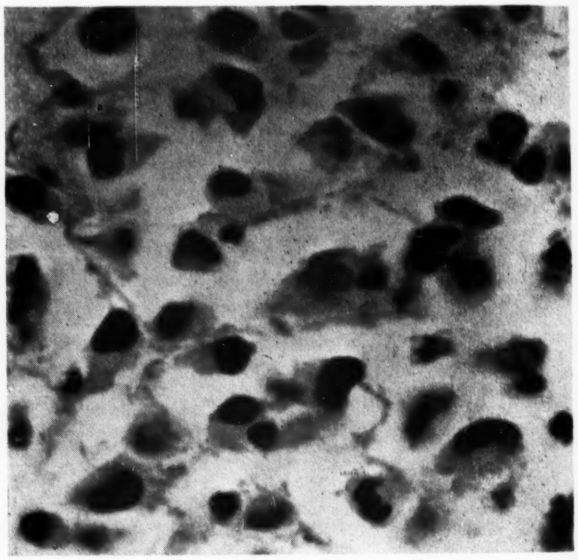

Fig. 4

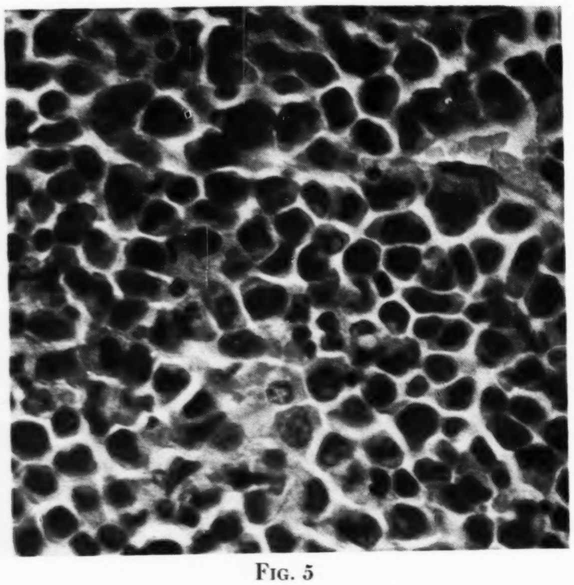

Fig. 5

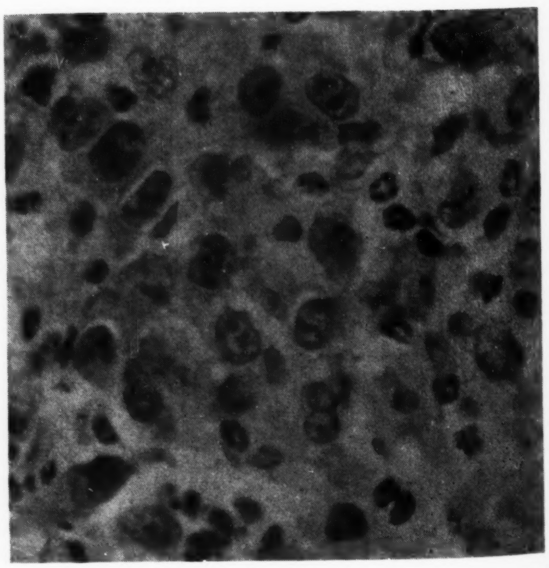

Fig. 6

hours gives 67 per cent "takes." The same strain when inoculated with tumor mince stored for 7 days shows a further fall to 41 per cent, and when the time of storage is 14 days, no tumors result.

Storage at $-30°$ C. results in a percentage of "takes" comparable to that seen in controls, although there appears to be a lengthening of the latent period with shorter periods of refrigeration (1–7 days). Storage at $-30°$ C. for long periods apparently produced some change in the tumor cells, since the sarcoma stored from 27 to 62 days showed a distinct shortening of the latent period. These tumors grew rapidly at first and then regressed, as noted in Table 1.

Tissue stored at $-70°$ C. for various times showed essential y the same average latent periods as controls, as well as similar percentages of "takes." The two groups showing a slightly lower percentage of tumors probably represent variations which are not significant and may be related to the relatively small numbers of animals used, as compared to the controls. In only one group of animals were regressions noted, and the occurrence of this phenomenon was confirmed by the biopsy of two tumors that subsequently regressed. The same is true for tumor tissue frozen at $-190°$ C. and stored at $-70°$ C.

The data in this table would indicate, therefore, that refrigeration for periods of time in excess of 14 days results in some changes in the tumor cells which are manifested in some instances by a regression after an initial period of growth.

The effect of refrigeration on the morphological characteristics of Sarcoma 37.—A representative area of the original Sarcoma 37 is shown in Figure 1. The tumor consists of ovoid and spindle cells with large nuclei showing a fairly marked variation in size and shape; many of the nuclei are vesicular, while others are densely hyperchromatic. Mitoses are numerous and average 2–4 per high-power field.

Representative sections of the material frozen at $-30°$ C. and used as the inoculum for the experiments in Group C, Table 1, are shown in Figure 3. There is a remarkable degree of preservation of tumor cells and a striking similarity to the unfrozen tumor material shown in Figure 1.

In contrast, tumor material stored at 0° C. for 24 hours shows many small pyknotic nuclei as well as large, pleomorphic cells (Fig. 2).

Figure 4 shows the state of the tumor frozen at $-70°$ C. and used as inoculum for the experiments in Group D, Table 1. While the nuclei are here also well preserved, there are somewhat fewer mitoses. In some instances, there was a shrinkage of the cytoplasm. The loose arrangement of cells in this photograph is due to the fact that the slide was prepared from a mince.

Figure 5 represents tumor material frozen in liquid nitrogen ($-190°$ C.) While where is some diminution in the size of the tumor cells, they appear in a remarkably good state of preservation. Many of the nuclei show mitotic figures, and there is little pyknosis and karyorrhexis.

Despite the minor variations in morphological characteristics resulting from exposure to different degrees of refrigeration, the resultant tumors, following mouse inoculation, were strikingly similar. Figures 6, 7, and 8 show typical fields of tumors resulting from the injection of material subjected to the three different temperatures. All show large, pleomorphic tumor cells with well preserved hyperchromatic and vesicular nuclei. Mitotic figures were frequently found.

Serial short interval studies of transplanted frozen and unfrozen tumor.—To study more closely the fate of transplanted tumor cells, minces of frozen and unfrozen sarcoma tissue were inoculated subcutaneously and the tumor tissue removed for microscopic study at 2-hour intervals for the first 8–10 hours, and at approximately 12-hour intervals thereafter until palpable tumors developed.

There was essentially no difference in the development of tumors from unfrozen and frozen tissue, except that in transplants of the latter, proliferation of tumor cells was slower than in the former. Two to 4 hours after inoculation, necrosis of the cells was marked in both groups, but viable-appearing cells were also present (Figs. 9 and 10). Within the next 4–6 hours almost all the tumor

FIG. 1.—Section shows the original Sarcoma 37 received from the National Cancer Institute. The tumor is a pleomorphic one, showing marked variation in nuclear size and numerous mitoses. Mag. approx. 500×.

FIG. 2.—Section shows Sarcoma 37 after exposure to 0° C. for 24 hours. There is a mixture of small pyknotic nuclei and many pleomorphic cells with large hyperchromatic nuclei. Mag. approx. 200×.

FIG. 3.—Section shows Sarcoma 37 after exposure to $-30°$ C. for 24 hours. Section is similar to that shown in Figure 1. Arrow indicates a cell in mitotic division. Mag. approx. 500×.

FIG. 4.—Section is of a tumor mince after exposure to $-70°$ C. for 24 hours. There are a few scattered cells with pyknotic nuclei and many cells with large hyperchromatic nuclei. Mag. approx. 500×.

FIG. 5.—Section is of tumor after exposure to $-190°$ C. for 24 hours. The tumor cells appear well preserved. Mag. approx. 200×.

FIG. 6.—Tumor 15 days after inoculation. Inoculum previously exposed to $-30°$ C. for 24 hours. Note numerous well preserved tumor cells with vesicular nuclei. Mag. approx. 500×.

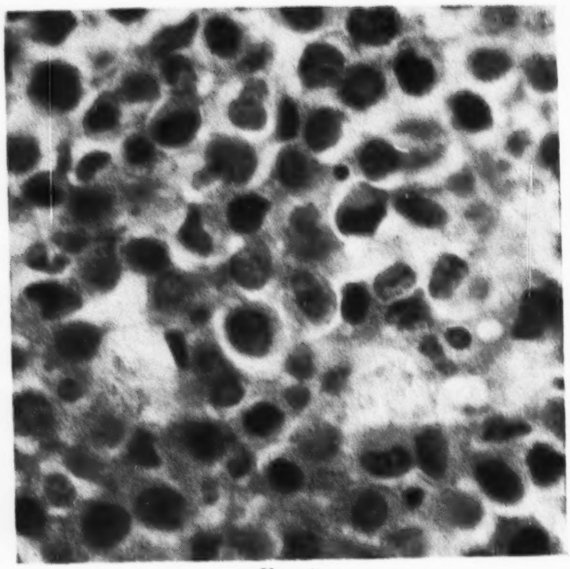

Fig. 7

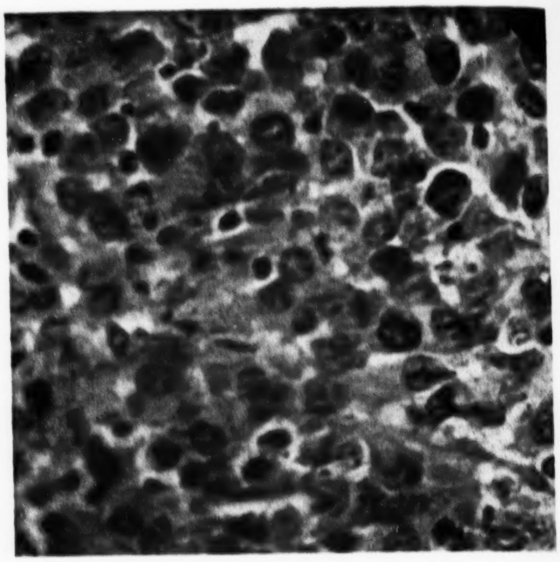

Fig. 8

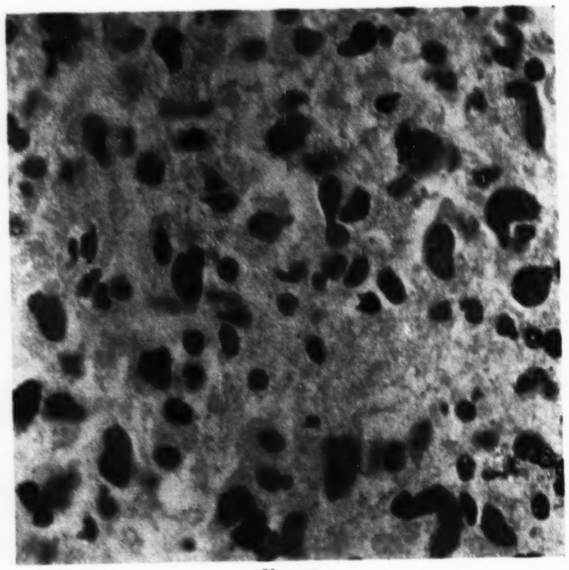

Fig. 9

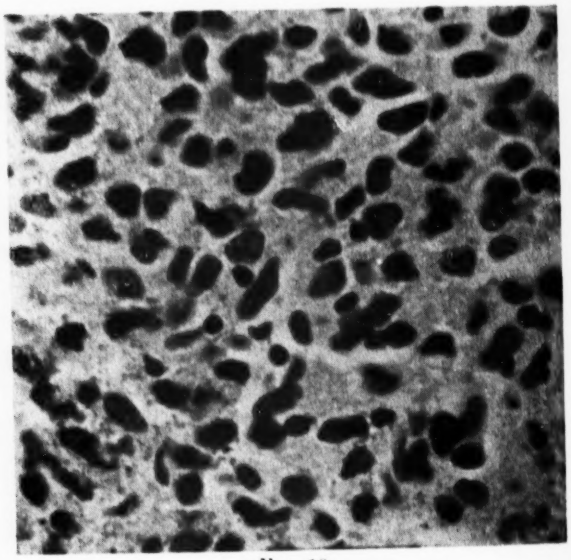

Fig. 10

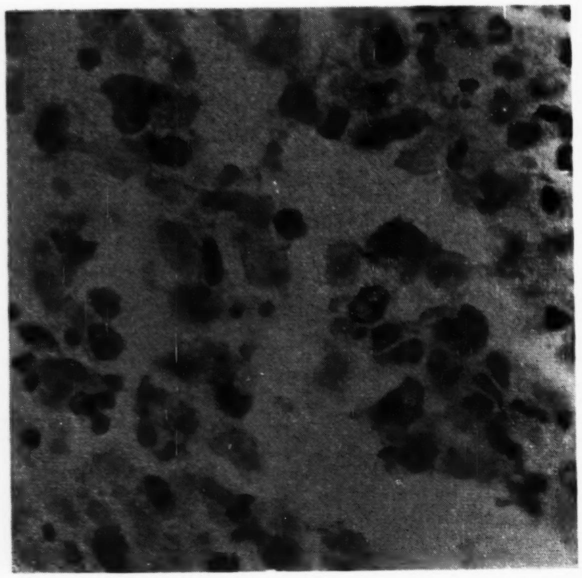

Fig. 11

Fig. 12

material became necrotic, but a few surviving cells were found at the periphery of the necrotic mass, on the surface of the fascia covering the abdominal muscles, or in the interstices between striated muscle cells. The latter localization was apparently due to the perforation of the fascia in the course of the inoculation. During the next 6–12 hours there was little change in development.

Approximately 24 hours after the implantation of untreated tumor tissue, cells begin to proliferate into the necrotic mass (Fig. 11). Extension of a thin sheet along the surface of the fascia and penetration between muscle fibers have also been observed. A similar process occurs in transplants of frozen tumor material. However, a developmental stage comparable to the controls was reached 48 hours after transplantation (Fig. 12). Control tumor transplants develop grossly palpable tumor nodules and show extensive growth microscopically 72 hours after transplantation (Fig. 13). During the same period, there is only very slow proliferation of frozen tumor transplants (Fig. 14). The transplants of frozen tumor show a lag period of approximately 2–3 days as compared to the controls.

The effect of repeated freezing and thawing on the transplantability of Sarcoma 37.—Many attempts were made to obtain tumor takes following repeated freezing at −30° C. and −190° C. and thawing to room temperature (approximately 20° C.). Tumor growth did not occur after two or more exposures to this treatment. The results of one such experiment are shown in Table 2.

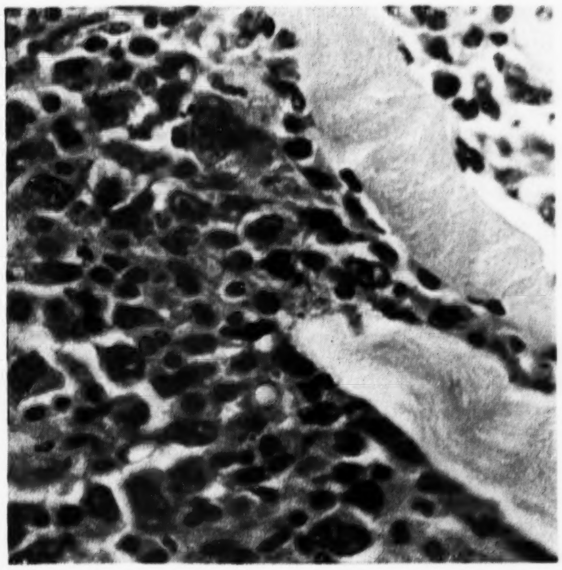

FIG. 13.—Unfrozen tumor 78 hours after inoculation. A continuous tumor mass has formed and is invading between striated muscle fibers. Mag. approx. 500×.

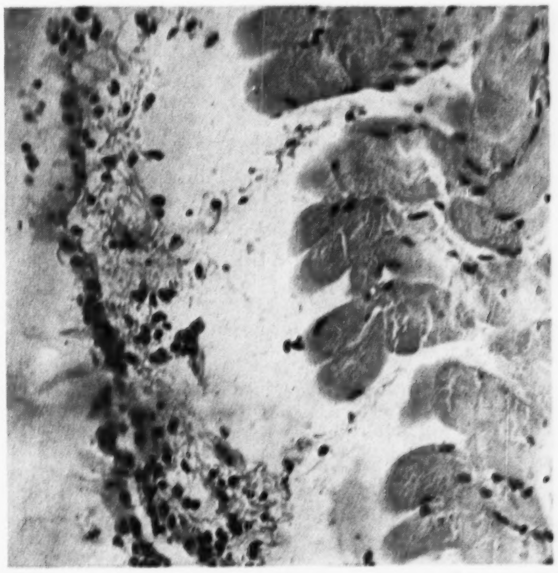

FIG. 14.—Frozen tumor (−70° C. for 24 hours), 70 hours after inoculation. Note clusters of tumor cells growing over the surface of fascial connective tissue. Mag. approx. 100×.

The effect of a single refrigeration of Sarcoma 37 on subsequent tumor generations.—During the course of these experiments it was noted that in some series the tumor transplants would fail to grow after several transplant generations. It was at first thought that this might be due to a developing resistance to the tumor by some of the strains, and accordingly tumor transfers between various strains were also carried out. The experi-

FIG. 7.—Tumor 15 days after inoculation. Inoculum exposed to −70° C. for 24 hours. Appearance is essentially the same as Figure 6. Mag. approx. 500×.

Fig. 8.—Tumor 14 days after inoculation. Inoculum exposed to −190° C. for 15 minutes and then at −70° C. for 24 hours. Appearance is essentially the same as in previous two figures. Note mitotic figure in center of field. Mag. approx. 200×.

FIG. 9.—Unfrozen tumor, 4 hours after inoculation. There are many small pyknotic nuclei, but scattered through the inoculum are large hyperchromatic nuclei.

FIG. 10.—Frozen tumor (−70° C. for 24 hours), 4 hours after inoculation. The inoculum appears similar to that noted in previous figure. Mag. approx. 500×.

FIG. 11.—Unfrozen tumor, 24 hours after inoculation. There are small pyknotic nuclei and a few polymorphonuclear leukocytes, but tumor cells with larger nuclei are also present. Mag. approx. 500×.

FIG. 12.—Frozen tumor (−70° C. for 24 hours), 48 hours after inoculation. A large area of necrotic tumor is present in the center of the figure. Arrow shows a cluster of tumor cells with large hyperchromatic nuclei at the periphery enmeshed in several muscle fibers. Mag. approx. 100×.

mental data indicated that a single freezing may have had some effect on the tumor which lowered its growth potential, so that it fai ed to take after several subsequent transfers. The data in Table 3 would tend to substantiate such an interpretation. Unrefrigerated tumor was carried successfully through 24 transplant generations in Rockland mice, at which time it was thought that refrigerated tumor would serve as well as unfrozen tumor, and this latter line of tumor transfer was therefore terminated. However, sarcoma material, derived from several transplant generations of unfrozen tissue which had been frozen at −30° C. and subsequently transplanted into the same strain, died out after only four transplant generations. Likewise, unfrozen tumor material carried for a single generation in CaF-1 and CFW and then frozen under the conditions stated in Table 3 were subsequently implanted into these respective strains and died out after six to seven transplant generations. Similarly, unrefrigerated tumor which had been transplanted only once in an unfrozen state in St L.U. mice, when refrigerated and subsequently carried through several transplant generations, died out after only five transplant generations. Controls are lacking in the last four groups because of the peculiar conditions under which this phenomenon was noted. However, this tumor has been carried in a fresh state through an indefinite number of generations in these and other strains by many investigators and is known to have low strain specificity. The data in Rockland mice alone substantiate the validity of this interpretation.

TABLE 2

EFFECT OF MULTIPLE FREEZINGS AT −190° C. AND THAWING ON THE TRANSPLANTABILITY OF SARCOMA 37

No. freezings and thawings	No. mice	Latent period	Percentage takes
1	17	14 days	100
2	10		0
3	5		0
4	5		0
5	5		0
6	5		0

TABLE 3

THE EFFECT OF A SINGLE REFRIGERATION OF SARCOMA 37 ON SUBSEQUENT TUMOR GENERATIONS

Strain	Conditions of refrigeration	No. of successful transplant generations
Rockland	Unrefrigerated controls	24
Rockland	−30° C. for 24 hours	4
CaF-1	−30° C. for 7 days	7
CFW	−70° C. for 24 hours	6
Combination	−30° C. for 24 hours	5
Combination	−70° C. for 24 hours	5

DISCUSSION

Loeb (26) has shown that, like normal cells, tumor cells also contain individuality and species differentials. The presence of such differential substances limits serial transplantation, in general, to the species in which the tumor arises; within that species successful transfer of tumor material is usually restricted to animals with the same genetic constitution. In some instances, however, the growth potential of the malignant cells is sufficiently high to overcome the deleterious effects resulting from genetic differences. Sarcoma 37 is apparently such a tumor, since it was transferred successfully for at least several generations in the four strains of albino mice used in these experiments, after having been received from the National Cancer Institute in mice of one of these strains (CFW), in which it had been carried for many transplant generations.

Transplantation of fresh tumor material was also carried successfully through 24 transplant generations in Rockland mice, at which point this phase of the work was voluntarily terminated. Whether or not transfer of such unfrozen material could have been carried through numerous transplant generations in the other two strains was not determined. However, as tumor material once frozen, it failed to survive after five to seven transplant generations in three of the strains and after four such transplant generations in Rockland mice.

A characterization of the growth potential of a tumor along these lines becomes important in the choice of mater'al with which one attempts the demonstration of a hitherto undiscovered virus. Some investigators believe that most, if not all, tumors may be of virus etiology. Failure to demonstrate virus has, among other factors, been attributed to rigid genetic specificity and specificity of cell type. A connective tissue tumor of relatively low genetic specificity, as Sarcoma 37 appears to be, might therefore be a favorable material for demonstrating a virus etiology.

Gye, Craigie, Mann, and co-workers believe they have demonstrated that cancer is caused by a virus, or, at least, by a similar active agent which is capable of starting a tumor when introduced into an animal. They base this conclusion on the following observations:

1. Tumor tissue may be frozen to extremely low temperatures (−79° C.) and subsequently transplanted successfully. Transfers of such material examined at short intervals after transplantation

fail to show evidence of surviving tumor cells, although the authors admit the impossibility of examining every cell in the implanted material. They conclude that the tumor cells, therefore, fail to survive freezing.

2. Normal mouse embryo tissue minces, when injected subcutaneously into inbred mice of the same strain, develop into relatively benign teratomas. Frozen mouse embryo tissue fails to develop into such a neoplasm.

3. Frozen-dried tumor tissue is also capable of producing tumors when inoculated into mice. No cellular structure was observed on microscopic examination of the inoculum.

4. A sarcoma suspension frozen in both 40 per cent dextrose and 40 per cent glycerol was active after 253 days.

The present investigations, as well as observations of other workers, are at variance with this interpretation. There are several investigations which indicate that normal as well as malignant tissues of warm-blooded animals may survive freezing to extremely low temperatures and possibly even lyophilization. In a previous report (3) we have cited these investigations, showing the survival of normal cells after freezing, as manifested by successful subcutaneous autotransplantation, and have further shown that an intact organ such as the thyroid and parathyroid gland may also be successfully autotransplanted following such treatment. To these examples one should also add the successful grafting into cutaneous tissues of frozen-thawed mouse (8) and human skin (40) and, in one instance, an apparently successful graft of this type with lyophilized human skin (40). Recently, Strumia (38) has also reported that human erythrocytes may survive freezing to extremely low temperature. As to embryonal tissues, Hetherington and Craig (20) have shown that chick embryo heart tissue will survive following exposure to temperatures between $-7°$ C. and $-70°$ C. for only a few hours, but embryonic tissues placed at 0° C. will survive for 300–400 hours after thawing; these investigators used growth in tissue culture as a criterion of viability. On the other hand, Mann (29) was unable to demonstrate survival of mouse embryo exposed to 4° C. Hirschberg and Rusch (21) and Andrewes (2) have recently summarized reports of successful and unsuccessful attempts to grow tumor as well as adult and embryonal tissue after freezing. It is probable that different species of embryonal tissue of homotherms vary in their sensitivity to cold, just as different organs in the same animal vary in their reaction to exposure to low temperatures.

Several observations in the present experiments bear on the question of whether or not tumor cells also survive after low temperature refrigeration. Tumor refrigerated at 0° C. became progressively less potent in the production of tumors as the duration of exposure to cold was increased; refrigeration for more than 7 days resulted in no successful tumor transfers. When freezing was carried out at lower temperatures, the percentage of "takes" was generally higher than at 0° C. The latent period in experimental and control groups varied, for the most part, between 10 and 15 days, with occasionally slightly longer or shorter periods in some groups. The freezing, therefore, apparently had no significant effect on the duration of the latent period. These various observations, while not conclusive to the argument, are better accounted for on the basis of cell survival than on the presence of a virus.

The regression of some tumors resulting from the implantation of frozen material and the apparent loss of potency of tumor once frozen, after several subsequent serial passages carried out in an unfrozen state, indicate either the development of an anti-genicity, a change in the potency of a virus (perhaps in the nature of a mutation), or some change in the character of the frozen tumor cell. These observations are also not conclusive to the present argument, but bear a relation to certain possibilities to be mentioned below. They indicate that, in testing the effects of various physical and chemical agents on transplantable tumors, it becomes necessary to carry the tumor through several transplant generations after the one during which the agent has been administered, in order to determine delayed effects of the procedure.

Other observations are more directly related to the problem of a viral etiology of tumors. Sections of tumor taken after freezing, but before transplantation, show well preserved tumor cells with numerous mitotic figures. Whether or not the latter indicate cell activity even at low temperatures, or simply a fixed state of cells which were in mitotic activity at the time freezing was carried out, remains to be determined. Gye, Begg, Mann, and Craigie (18) have stated that examination of tumor after passage through a mincer shows that a majority of the cells were damaged. According to these investigators after simple freezing there are even fewer apparently intact cells. Mann and Dunn (30) have stated that frozen-dried material shows no cellular structure. These observations are at variance with the present results in which sections of frozen pieces of tumor, as well as of minces, show most of the tumor cells to be well preserved. As for the frozen-dried tumor, the observations do not agree with results generally obtained in speci-

mens prepared for histological study by the freezing-drying technic first suggested by Altmann (1) and later modified and improved by Gersh (16). In recent unpublished experiments we have noted numerous well preserved tumor cells after lyophilization. Furthermore, rehydration before histological examination might have revealed preserved tumor cells in the experiments of Mann and Dunn.

However, histologically preserved cells do not necessarily indicate viability; freezing might well result in fixation of cytological elements rather than preservation in a viable state. On the other hand, the serial time-interval studies indicate the presence of viable cells in the inoculum. It should be remembered that in the inoculation of a tissue mince, or in any homoiotransplantation of normal tissues, as shown by Loeb (26), a large part of the inoculum or transplant undergoes early central necrosis. The surviving peripheral cells then multiply (tumor) or regenerate (normal tissue). The tumor cells in both unfrozen and frozen material which reach spaces between mucle fibers appear to be protected from the initial necrosis which involves the bulk of the inoculum. These protected cells subsequently multiply and produce the tumor mass.

With the exception of Cramer (12) and, more recently, Gye *et al.* (18), all investigators have attributed this phenomenon to surviving tumor cells. The evidence for survival of tumor cells rather than transfer of virus rests essentially on the following observations:

1. Surviving tumor cells could be demonstrated by early serial time-interval studies as in the present experiments.

2. Klinke (23–25) observed that some frozen tumor cells, as well as frozen embryonal heart tissue cells of the fowl, show positive vital staining with neutral red after thawing. In some experiments tissues were frozen to as low as $-253°$ C. He was also able to show survival of interstitial cells of the cock and rabbit testis by this method.

3. Breedis and Furth (6) have observed that doses of x-ray which kill tumor cells at room temperature also destroy the ability of transplanted frozen tumor material to develop tumors. These dosages do not inactivate known viruses at room temperature, and it would not be expected that they would act differently at low temperatures.

The observations with repeated freezing and thawing in the present experiments are in accord with several previous investigations, including those of Mann (28). The various reports differ only in the number of such repetitions before successful transfer can no longer be made. Sarcoma 37 appears to be one of the more sensitive tumors, since a second freezing-thawing fails to result in tumor growth. Luyet and Gehenio (27) have shown that in normal tissues repeated freezing and thawing will eventually result in the death of all cells. On the other hand, Mann (28) states that certain viruses are also sensitive to such treatment. In general, the eventual complete loss of potency by repeated freezing and thawing is more consistent with the interpretation that the effectiveness of frozen material is due to the survival of tumor cells, since many infectious viruses can survive an indefinite number of such treatments.

Furthermore, the observation that lyophilized tumor material can be successfully transferred does not el'minate the likelihood that living cells are present in the inoculum. In addition to the sing'e obervation by Webster (40) that parts of a lyophi ized skin graft may survive, the investigations of Luyet and Gehenio have shown that dehydration facil tates rather than impedes the survival of frozen cells. There is some indication that the differences in results obtained by rapid and slow freezing, which have been noted in the introduction, are explainable on the basis of changes in either the volume or physical state of the water content within the cells. Luyet and Gehenio contend that rapid freezing is essential to the survival of cells unless there is a previous or concomitant dehydration of the tissue. Breedis (5) explains the favorable results in tumors with slow freezing on the theory that ice crystals form in the interstitial tissue where an osmotically active area is set up and causes dehydration of the intracellular protoplasm. The most rapid rates of freezing used by most investigators, including Breedis and Furth, are not sufficient to produce an amorphous or vitreous state; to accomplish this, Luyet and Gehenio state that the rate of freezing of the tissue must be at least several hundred degrees per second. An intermediate rate of freezing, between that regarded as slow freezing and the rapid rate as defined by Luyet and Gehenio, is the one which most investigators designate as rapid freezing. The latter may perhaps not produce an effective interstitial osmotically active area. Despite these differences, it appears that dehydration of the intracellular protoplasm can protect cells against the injurious effects of freezing, and the use of 40 per cent glucose or 40 per cent glycerin probably facilitates dehydration.

These experiments do not rule out the presence of a transmissible factor as a "continuing cause" of cancer. They do indicate, however, that the criteria used by Gye and co-workers are not sufficiently critical to eliminate the possibility of a transfer of viable cells. The term "virus" has in re-

cent years become increasingly difficult to define, since pathogenic viruses, latent viruses, and self-duplicating cytological constituents of normal cells appear to have many points of similarity. The criteria ordinarily employed in defining viruses were developed in investigations dealing with parasitic organisms. These criteria apply to the experimental transmission of relatively few tumors, and in these there is generally no history of a natural transmission. The "continuing cause" of every type of cancer may be an intracellular organizing element which in some instances may be readily separated from other cellular elements and introduced into new cells with resulting tumor production. The failure to isolate such an element from the great majority of mammalian tumors may be due only to limitations of technic.

Further work is in progress on the behavior of normal and tumor tissues in homoio- and hetero-transplants, after freezing and dehydrating, since it is believed that the problems raised by preliminary work may be of basic importance.

SUMMARY

The effects of low temperatures (0° C., −30° C., −70° C., and −190° C.) on Sarcoma 37 of mice have been studied. In general, freezing does not appreciably alter the latent period of tumor transplants nor their morphological characteristics, either before transplantation or after the development of tumors. Exposure to 0° C. results in a percentage of "takes" which is inversely proportional to the duration of exposure, but exposure to lower temperatures results in as high a percentage of successful transplants as controls regardless of the duration of exposure used in these experiments.

Exposure to temperatures lower than 0° C. alters the tumor cells in some way as evidenced by (*a*) regression of some tumors after an initial period of growth and (*b*) a loss of viability of once-frozen tumor material after several subsequent passages in an unfrozen state. Repeated freezing and thawing also results in a loss of viability of tumor cells.

Serial short time-interval studies of frozen and unfrozen tumor transplants indicate a survival of transplanted cells in both groups.

These findings are discussed with respect to the concept of virus as the "continuing cause" of tumor formation.

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Announcements

MEETING OF SOUTHWESTERN SECTION FOR CANCER RESEARCH

The first meeting of the Southwestern Association for Cancer Research was held on May 7, 1948, at the Medical Branch of the University of Texas in Galveston. It was agreed that the purpose of the society was to encourage cancer research and the exchange of information between cancer workers in Arkansas, Louisiana, Oklahoma, and Texas. Full membership was limited to those who were also members of the American Association for Cancer Research, and the officers were directed to apply for affiliation as a regional section of the National Society. There were eighteen persons present when the regional association was organized, and charter membership included 9 members and 31 associate members. They elected Dr. Jacob Furth as president and Dr. W. A. Selle as secretary-treasurer, both of whom were largely responsible for organizing the meeting. An informal presentation of research in progress was given by those present following the business meeting.

The School of Aviation Medicine, Randolph Field, Texas, was host to the society at the next meeting, November 5, 1948. A program of scientific and clinical papers was given at that time. Most of the members also attended the meetings of the Society for Experimental Biology and Medicine which followed. The Spring Meeting on May 20, 1949, was held at the M. D. Anderson Hospital for Cancer Research in Houston, and some of the members participated in the Symposium on the Fundamentals of Cancer Research which was sponsored by that institution. Officers elected for the following year were Dr. Bela Halpert, president, Dr. Charles Spurr, vice-president, and Dr. Walter J. Burdette, secretary-treasurer.

Two meetings were also held the following year. The meeting in Oklahoma City on November 11, 1949, was sponsored by the University of Oklahoma School of Medicine. In addition to the regular program of scientific papers and discussion, Dr. Stanley P. Reimann addressed the membership at a luncheon given by the Oklahoma Division, American Cancer Society. Both this and the meeting on May 5, 1950, at the Veterans Administration Hospital in Houston were held immediately before that of the Society for Experimental Biology and Medicine. At the last meeting new officers elected for the coming year were Dr. C. P. Oliver, president, and Dr. Mark R. Everett, vice-president.

The American Association for Cancer Research accepted the Southwestern Section as the first regional affiliate at the annual meeting in 1950. At the present time the organization has 19 full members and 72 associate members.

INTERNATIONAL CANCER MEETINGS

The Transactions (1,411 pages) of the Fourth International Cancer Congress held in St. Louis in September, 1947, have been published in ACTA, Unio Internationalis Contra Cancrum in 1948, 1949, and 1950. They can be obtained by sending a postal money order for $25.00, made payable to ACTA, 61 Voer des Capucins, Louvain, Belgium.

The Transactions of the meeting of the International Cancer Research Commission in Paris, July, 1950, have been published in ACTA, Vol. VI, No. 8 (35 pages).

The Semaine d'Étude sur le Problème Biologique du Cancer, organized by Professor R. Rondoni and held by the Pontifical Academy of Science in the Vatican, June, 1949, has been published and constitutes a volume of 348 pages. Information can be secured from Dr. Pietro Salviucci, Chancellor of the Academy, Vatican City.

Transactions of the meetings of the International Cancer Research Commission, Dr. W. U. Gardner serving as U.S. representative, and of the Fitch International Cancer Congress held in Paris in July, 1950, under the presidency of Professor A. Lacassagne, will be published in ACTA, with Professor J. Maisin continuing to serve as Editor-in-Chief. The Commission will meet in December, 1951, at Lisbon and in December, 1952, at Bombay, both under the chairmanship of Professor V. Khanolkar, Director of Laboratories, Tata Memorial Hospital, Bombay. The Sixth International Cancer Congress will be in Brazil in the summer of 1953 under the presidency of Professor A. Prudente, Benjamin Constant 171, São Paulo.

The Council for the Co-Ordination of International Congresses of Medical Sciences (C.C.I.C.M.S.), under the presidency of Professor J. Maisin, conducted a symposium on "Geographic Pathology and Demography of Cancer, July 29 to August 14, 1950, in Regent's Park College, Oxford. Information can be obtained, from Professor Maisin, 61 Voer des Capucins, Louvain, Belgium.

OAK RIDGE INSTITUTE OF NUCLEAR STUDIES

The eighteenth, nineteenth, and twentieth courses in the technic of using radioisotopes in research will be given by the Special Training Division of the Oak Ridge Institute of Nuclear Studies during the winter and spring of 1951. Dates for the courses are as follows: January 8 to February 2; February 19 to March 16; and April 16 to May 11.

The courses are designed to acquaint research workers with the safe and efficient use of radioisotopes in research. The course work consists of laboratory work, lectures on laboratory experiments, general background lectures and special topic seminars. Experiments are conducted covering the use and calibration of instruments, the purification and separation of radioactive materials from inert and other radioactive materials, measurement and use of Carbon 14, pile activations, radioautographs, etc. Seminars include such topics as the use of radioisotopes in animal experimentation, use of radioisotopes in humans, principles and practice of health physics, design of radiochemical laboratories, effect of radiation on cells, and similar topics.

The Special Training Division can accommodate thirty-two participants at each of the three courses. A registration fee of $25 is charged, and participants will bear their own living and traveling expenses.

Additional information and application blanks may be obtained from Dr. Ralph T. Overman, Chairman, Special Training Division, Oak Ridge Institute of Nuclear Studies, P.O. Box 117, Oak Ridge, Tennessee.

BERTNER LECTURESHIP

There was announced at the Fourth Annual Symposium on Fundamental Cancer Research of the University of Texas M. D. Anderson Hospital for Cancer Research, held at the Shamrock Hotel on May 12, the establishment of an annual lectureship for the Symposium by the Bertner Foundation, of Houston. The lectureship is to cover the most significant single advance in the field of cancer research, usually for the previous year. The selection of the lecturer will be made from a special nominating committee of persons from the basic scences, i.e., physics, chemistry, biology, and medicine. Names selected will be presented to a special Awards Committee representing the Texas Medical Center, the M. D. Anderson Hospital for Cancer Research, and the University of Texas. A medallion signifying the Bertner Foundation Award and lectureship will be given with the honorarium.

There was also announced a reciprocal exchange senior fellowship program with the Memorial Hospital Center for Cancer and Allied Diseases, in New York, from the Houston Endowment Foundation by Jesse H. Jones, president of the Foundation. The grant provides that each fellowship will be known as "The Jesse H. Jones Fellowship in Cancer Education Honoring Dr. E. W. Bertner." Each annual fellowship stipend is not to exceed $5000.00 and provides for yearly renewals for a total period not to exceed 3 years.

AMERICAN CANCER SOCIETY GRANTS FOR SCHOLARS IN CANCER RESEARCH

The American Cancer Society in 1951 will inaugurate a program to help newly trained scientific scholars establish themselves in the field of cancer research. The Grants for Scholars in Cancer Research, as the program will be known, are designed to bridge the gap between the completion of fellowship training and the period when the scientists has thoroughly demonstrated his competence as an independent investigator. A limited number of American Cancer Society Scholars will be appointed annually on recommendation of the Committee on Growth of the National Research Council. A grant of $18,000, payable over 3 years, will be made directly to each Scholar's institution by the American Cancer Society as a contribution toward his support or his research, or both.

Medical schools, hospitals, research institutes, and other institutions with a primary or substantial interest in cancer research are invited to submit applications for these grants. Applications for grants to be effective July 1, 1951, should be submitted prior to January 1, 1951. Inquiries or requests for application forms should be addressed to the Executive Secretary, Committee on Growth, National Research Council, 2101 Constitution, N.W., Washington 25, D.C.